# CHARACTERISATION OF THE IMMUNE RESPONSES OF RUMINANTS AND MICE TO THE WELGEVONDENSTOCK OF COWDRIA RUMINANTIUM

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# DEDICATION

**To my dear wife Margaret Kobilo Chingi and the children Kipruto Chepkangor, Kangogo Kiprop, Kibet Kiptalam, Jerotich, Jelagat, Jepkorir and Kimichingi for their love, support. and patience**

# DECLARATION

The study presented in this thesis was composed by myself. I declare that the work in it was performed by myself and any work performed by other persons is specifically stated in the text and in the acknowledgement section.

**Alfred Chingi Kibor**

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# ABBREVIATIONS

| | | |
|---|---|---|
| BSA | = | bovine serum albumin |
| BAC/BAE | = | Bovine arterial cells/bovine endothelial cells |
| $ddH_20$ | = | double distilled water |
| DMSO | = | Dimetylsulfoxide |
| EBs | = | Elementary bodies |
| FCAS | = | Flourescein activated cell scanner |
| GMEM | = | Glasgow Minimum Essential Medium |
| HEPES | = | (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] |
| Hsp | = | Heat shock protein |
| IEBs | = | Inactivated elementary bodies |
| IM | = | intramuscular |
| IPTG | = | Isopropyl-b-thiogalactopyranoside |
| I/T | = | Infection treatment |
| L | = | litre |
| M | = | molar |
| mM | = | milimolar |
| mg | = | milligram |
| ml | = | mililtre |
| NC | = | nitrocellulose |
| PBS | = | Phosphate Buffered Saline |

| | | |
|---|---|---|
| PBST | = | Phosphate Buffered Saline Tween 20 |
| PC | = | post challenge |
| PI | = | post inoculation |
| PM. | = | post mortem |
| PMSF | = | Phenylmethylsulphonyl fluoride |
| SC | = | subcutaneous |
| SDS-PAGE | = | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| μg | = | microgam |
| μl | = | microlitre |

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# THE UNIVERSITY OF EDINBURGH

# ABSTRACT OF THESIS

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Title of Thesis CHARACTERISATION OF IMMUNE RESPONSES OF RUMINANTS AND MICE TO THE WELGEVONDEN STOCK OF C. RUMINANTIUM

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*Cowdria ruminatium* is the causative agent of heartwater, an important tick-borne disease of ruminants in sub-Saharan Africa. Animals which recover after natural or experimental infections of heartwater are solidly immune to homologous challenge but, the immune responses responsible for protection against heartwater are poorly characterised.

This study sought to identify antigens involved in protective immune responses to *Cowdria* by Western blotting using immune sera and surface labelling of elementary body (EB) proteins using biotin to identify which of these are outer membrane proteins. Antigens which could be considered as potential vaccine candidates were identified.

Immunisation of goats with live *Cowdria* or with inactivated elementary bodies (IEBs) leads to development of antibodies to at least six antigenic components of the EB of 24kDa, 27kDa, 31kDa, 58kDa and 66kDa. In contrast immunisation of goats with detergent extracted soluble antigens stimulated production of antibodies to only four antigens of 24kDa, 27kDa, 28kDa and 31kDa. Six surface exposed antigens of the *Cowdria* elementary body were identified by biotin labelling, with molecular masses of 21kDa, 28kDa, 31kDa, 62kDa, 74kDa and 115kDa and are therefore considered outer membrane proteins. These proteins reacted with antibodies in sera raised by immunisation of goats with live or inactivated EBs. The 58kDa heat shock protein (GroEL) of C. ruminantium is an immunodominant antigen. The immune responses to 58kDa antigen expressed as a recombinant in *E. coli* were investigated by immunisation of mice and sheep. The immunised animals stimulated specific antibody which reacted with the native homologue on the EB 58kDa.

In order to investigate the possibility that immunisation with live or inactivated organisms induces qualitatively different immune responses , IgG isotyping was undertaken using sera from natural and experimental infections of *C. ruminantium* or immunisation with IEBs. Production of $IgG_1$ and $IgG_2$ antibodies in animals has been associated with T helper1 and T helper 2 (Th1 and Th2) responses respectively. Antigen specific $IgG_1$ but not $IgG_2$ was detected in the sera of goats and sheep experimentally infected with *C. ruminantium*.. In contrast a low but detectable concentration of $IgG_2$ and a high concentration of $IgG_1$ was detectable in field sera from cattle naturally exposed to heartwater. Specific $IgG_2$ was induced in goats after first priming the immune response by immunisation with IEBs. $IgG_1$ was dominant in sera of goats after immunisation with IEBs however, immunisation of cattle with killed organisms induced a high level of specific $IgG_1$ and $IgG_2$. Live infections of *Cowdria* in mice induce a dominant $IgG_2$a antibody response unlike the case in goats and sheep. This indicated that mice respond differently from small ruminants to live *C. ruminantium*. The results suggest that live organisms suppress a Th1 type antibody profile ($IgG_2$ production) except following immunisation with killed organisms. The suppression of protective Th1 responses of immunised goats aids the transmission of the parasite. Production of $IgG_2$ after challenge of IEB immunised goats may provide a method for monitoring vaccination trials in the future.

Immunisation with recombinant 58kDa heat shock protein of *C. ruminantium* led to development a of specific antibody response of the $IgG_1$ isotype. Challenge of immunised sheep showed a highly significant reduction ($p<0.005$) in infection rates of brain capillary endothelial cells in the immunised group compared to control animals. However the incubation period and clinical outcome were not significantly different in controls and immunised sheep. Mice immunised with recombinant 58kDa were partially protected against virulent homologous challenge. The lack of protection in sheep was attributed to failure of the antigen to induce a detectable lymphocyte response.

IFN-$\gamma$ is produced by PBMC from I/T and IEB immunised goats in response to stimulation with *Cowdria*. In contrast, no IFN-$\gamma$ response was detected with PBMC from sheep immunised with recombinant 58kDa hsp.

Protection of ruminants against cowdriosis in the future will depend on the development of save and easy to use vaccines. This study identified at least 6 antigens for further study to which dominant antibody responses were made by animals immunised with live or inactivated antigens. The use of alternative antigen delivery system for induction of T helper responses to *Cowdria* antigens are required for assessment of candidate vaccines for immunisation.

# CHAPTER ONE

# INTRODUCTION

## *1.1. Introduction*

Heartwater (Cowdriosis) is an infectious virulent tick-borne disease of domestic and wild ruminants caused by the rickettsial organism *Cowdria ruminantium.* The disease is characterised by high fever, nervous symptoms, hydrothorax and hydropericardium. Heartwater is transmitted transtadially by ticks in the genus *Amblyomma* (Uilenberg, 1983; Camus and Barre, 1988; Kobold, Martinez, Camus and Jongejan, 1992).

Heartwater occurs throughout most of sub-Saharan Africa from Cape Province of South Africa to Kassala in the Sudan, from Senegal in the west to Somalia in the East (Uilenberg, 1983). It occurs over an estimated area of 13 million $km^2$ (Neitz, 1968). The disease also occurs in the islands near the eastern coast of Africa such as Madagascar, La Reunion and Mauritius (Perreau *et al.,* 1980), Zanzibar (Flash *et al.,* 1990), Comoros (Du Plessis, Van Gas, Oliver, and Bezuidenhout, 1989), Sao Tome in the Atlantic Ocean (Uilenberg, Corten and Dwinger, 1982) and some Caribbean Islands (Uilenberg, 1983; Kobold, Matrinez, Camus and Jongejan, 1992). The American continent is now at risk because of the presence of suitable *Amblyomma* vectors on the mainland (Camus, Barre, Martinez and Uilenberg, 1996).

In East, Central and Southern Africa, heartwater is ranked as the third most important disease of livestock after east coast fever and the tsetse transmitted trypanosomiasis (Uilenberg, 1983). In these areas mortalities due to heartwater are as great as those caused by babesiosis and anaplasmosis combined (Neitz, 1968). North of the equator, it is ranked as the third most important livestock disease after rinderpest and schistosomiasis (Camus and Barre, 1988 quoting Provost, unpublished data). In terms of distribution, however, heartwater is more widespread than the other tick-borne diseases in that 175 million ruminants in Africa and 81,000 in the Caribbean are at risk (Norval, 1991).

Animals which recover from natural and experimental heartwater infection develop solid immunity to homologous challenge and show a variable degree of resistance to heterologous challenge. Immunity to heartwater in the ruminant is not clearly understood. Antibodies do not seem to play a significant role in protecting the animal against challenge. This has led to the belief that cell mediated immunity may be responsible for protection. In the mouse, protection is by $Lyt2^{+}$ ($CD8^{+}$) T cells (Du Plessis, Berche and Van Gas, 1991; Du Plessis, Gray and Van Strijp, 1992).

Control of heartwater has been achieved in three ways namely:-

1. Tick control by regular application of acaricides to kill the vector i.e. ticks in the genus *Amblyomma.*
2. Treatment of infected animals with chemotherapeutic compounds such as tetracyclines and sulphonamides.

3. Vaccination by the infection/treatment method. The vaccines used are either blood stabilates from infected animals or ground-up tick suspensions from infected tick larvae (Oberem and Bezuidenhout, 1987a). The vaccines are administered intravenously followed by treatment with antibiotics on the second day of fever.

These methods have worked well but they have limitations such as development of resistance to acaricides by the ticks and the high cost of both drugs and acaricides. Furthermore, the infection and treatment method of vaccination is severely limited by antigenic diversity among isolates of *C. ruminantium,* lability of vaccine therefore requiring cold storage, high cost of production and the fact that they can only be administered by a qualified person.

### *1.2. Economic Importance of Heartwater /Cowdriosis*

The economic importance of this disease has been reviewed by Camus and Barre (1988), Norval (1991) and Camus *et al.* (1996).

In 1990 FAO/WHO/OIE estimated the population of cattle in Africa and the Caribbean Islands to be 175 million and 81,000 head respectively. These animals are either in countries where heartwater has been confirmed (Provost and Bezuidenhout, 1987) or where vectors occur but the disease has not been confirmed (Walker and Olwage, 1987).

The economic effects due to heartwater fall into two main categories namely direct and indirect losses (Camus and Barre, 1988; Norval, 1991).

Direct losses are those which arise due to mortality which occurs under the following conditions: when susceptible animals are moved to endemic areas from heartwater-free areas, when there are epidemics of the disease, when the vectors spread from endemic areas to previously tick-free areas; when there is failure of tick control and finally when susceptible animals are raised in endemic areas.

Indirect losses arise from the following: subclinical infections which affect the productive performance of the animal, the cost of controlling the vectors, treatment of clinical cases and the inability to upgrade or improve local breeds by crossbreeding with exotic animals since, pure-bred exotic animals and their crosses are highly susceptible to this disease and always develop the peracute and the acute forms of the disease (Uilenberg, 1982b; Camus and Barre, 1988). Finally the export of animals from heartwater endemic countries is limited by legislation due to the risk of spreading the disease to free countries. Another danger the disease poses is that it could spread to areas where there are potential vectors like the North American continent (Uilenberg, 1983) and the Middle East.

The aim of the work described in this thesis is to study the immune responses of ruminants and mice to *C.ruminantium* by: characterisation of antigenic components of the elementary body of *C. ruminantium*, Investigation of the humoral and cellular immune responses of goats inoculated with live or inactivated immunogens and characterisation of immune responses of mice and sheep immunised with a recombinant 58kDa heat shock protein of *C. ruminantium.*

# CHAPTER TWO

# REVIEW OF THE LITERATURE

## *2.1. History.*

Heartwater was first recognised in South Africa in sheep and goats. The earliest record is from 1838 when Louis Trichardt (cited by Neitz, 1947) who reported a nervous disease in sheep resulting from tick infestation. The tick was later identified as *Amblyomma herbraeum* by Lounsbury (1900). Other early reports of the disease date back to 1858 (Alexander, 1931 quoting the evidence of Mr. Webb) and 1860 (Hutcheon, 1900). The disease was later reported in Central Africa (van Saceghem, 1918), Kenya (Daubney, 1930a), Malagasy (Durieux, 1931), the Sudan (Curasson and Deply, 1928) and the rest of East Africa (Lewis, 1939).

Although the disease was recognised in the mid 1880s it was not until 1925 that Cowdry (1925a, 1925b) demonstrated that the causative agent was a rickettsia-like organism present in tissues of infected animals and the vector. He gave it the name *Rickettsia ruminantium* which was later changed to *Cowdria ruminantium* by Moshkovskii (1947).

## *2.2. Identification and Classification of Cowdria ruminantium.*

### *2.2.1. Classification.*

Cowdry (1925a, 1925b) identified the heartwater organism as a rickettsia by its intracellular location, morphology and gram staining characteristics. Mohan (1968) and Prozesky, Bezuidenhout and Paterson (1986) examined *C. ruminantium*

in endothelial cells and concluded that morphologically were similar to chlamydia more than rickettsia. Du Plessis (1975), on the other hand observed that *Cowdria* were found freely in the cytoplasm of reticuloendothelial cells of ruminants as rickettsia. *Cowdria* however are normally found in membrane bound vacuoles in the cytoplasm of infected endothelial cells (Pienaar, 1970; Du Plessis, 1985b; Jongejan, *et al.*, 1991c). Although *Cowdria* and *Chlamydia* have similar life cycyles, they do not have close phylogenetic relationship. Scott (1987) gave the current taxonomical position of *C. ruminantium* as:

Order: Rickettsiales

Family: Ehrlichae

Genus: *Cowdria*

Species: *ruminantium*.

Analysis of the nucleotide sequence of the 16S ribosomal RNA gene of *C. ruminantium* shows that it is closely related phylogenetically to some members of the tribe *Ehrlichiae* (van Vliet, Jongejan and van der Zeijst, 1992).

### *2.2.2. Morphology and staining.*

Rickettsiae are fastidious obligate intracellular bacteria which are metabolically independent from their host cells (Bovarnick and Snyder, 1949). They are pleomorphic coccobacilli which range from 300 nm to 700 nm in size. They inhabit cytoplasmic vacuoles in the cells they infect. Colonies of organisms in the Family *Ehrlichiae* to which *C. ruminantium* belongs appear as compact inclusions containing varying number of organisms which occur in clusters referred

to as initial bodies (reticulate bodies) and elementary bodies (Ewing, 1969). These organisms are Gram negative, however they stain better with Giemsa (Woldehiwet and Ristic, 1993).

Organisms in the genus *Cowdria* range between 300 nm to 500 nm in diameter and are found in the cytoplasm of endothelial cells of the blood capillaries in the brain, renal glomeruli, jugular vein and in neutrophils and macrophages of infected ruminants (Cowdry, 1925a,b; Pienaar, 1970; Du Plessis, 1985a; Logan *et al.*, 1987b). The colonies of these organisms occur singly or in clumps morphologically, individual organisms are pleomorphic (Cowdry, 1925a, b; Jackson and Neitz, 1932; Du Plessis, 1970b). Pienaar (1970) described three forms of colonies of *C. ruminantiun* namely: small, medium and giant measuring 0.49 μm, 1.04 μm and 2.7 μm in diameter respectively. Other forms include ring shapes, bacillary, diploid and horse shoe (Andreasen, 1974; Ilemobade, 1976). Larger forms of up to 4.0 μm have been reported (Pienaar, 1970). Very small forms have also been described (Du Plessis, 1975a; Prozesky and Du Plessis, 1987). *Cowdria* has a life cycle similar to that of *Chlamydia* (Jongejan, *et al.*, 1991c). Its life cycle of infection is composed of elementary (EBs) which change into reticulate bodies in the host after infection and develop into EBs which mature during intracellular infection.

### *2.3. Isolates of Cowdria ruminantium.*

Several isolates of *C. ruminantium* have been described and are reviewed by (Bezuidenhout, Prozesky, Du Plessis and Van Amstel 1994) (Table 2.1) and

Camus, Barre, Martinez and Uilenberg, 1996). More stocks are being reported as research in heartwater continues for instance, the 10 stocks isolated recently from different parts of Kenya (Ngumi Rumberia, Williamson, Sumption, Lesan, and Kariuki, 1997).

Table 2.1 Stocks (strains) of *C. ruminantium*

| Stock | Source | Reference | Country of origin |
|---|---|---|---|
| Ball 3 | Cow | Haig (1952) | S. Africa |
| Breed | Angora goat | Du Plessis *et.al. (*1983) | " |
| Comoro | *A. variegatum* | Du Plessis *et.al.* (1989) | Comoro |
| Germishuys | Sheep | Du Plessis *et. al.* (1984) | S. Africa |
| Kumm | Mice | Du Plessis & Kumm (1971) | " |
| Kwanyanga | Mice | Mackenzie & van Rooyen (1981) | " |
| Nonile | *A. herbraeum* | Mackenzie & McHardy (1984) | " |
| Mara | *A. herbraeum* | | " |
| Welgevonden | Mice | Du Plessis (1985a) | S. Africa |
| Mali | *A. variegatum* | Logan *et.a*l. (1985) | Mali W. Africa |
| Gardel | Goat | Uilenberg, *et. al.* (1985) | Guadeloupe |
| UmBanein | Goat | Abdel Rahim & Shomein (1978) | Sudan |
| Kiswani | *A. variegatum* | Kocan *et.al.* (1987) | Kenya |
| D225 | Goat | Ilemobade, (1976) | Nigeria |
| Palm River | *A. herbraeum* | Byrom&Yunker (1990) | Zimbabwe |
| Highway | " | " | " |
| Silver Springs | " | " | " |
| Kenya isolates | | Ngumi *et al.* (1997) | Kenya |

## 2.4. Life Cycle of *Cowdria ruminantium.*

### *2.4.1. Life cycle in the tick vector.*

*Cowdria ruminantium* parasitises epithelial cells of the midgut (Cowdry, 1925b; Kocan and Bezuidenhout, 1987), malphigian tubules, epithelial cells and acini of salivary glands (Kocan, Bezuidenhout, and Hart, 1987) and haematocytes (Du Plessis, 1985a) of its tick host. Colonies have been seen in larval, nymphal and adult stages of the ticks (Kocan, et al., 1987; Kocan, Morzaria, Voigt, Kiarie and Irvin, 1986). These colonies are invariably in membrane-bound intracellular vacuoles regardless of their location in the host (Bezuidenhout, 1984).

Kocan *et al.* (1986) described the life cycle of *C. ruminantium* in *A. variegatum* under experimental conditions. Colonies appeared 15 days after the tick had engorged and dropped off the mammalian host. The size of colonies in the midgut epithelial cells is variable, the predominant stage of the organism within the cells being the reticulated form and reproduction was by binary fission. In addition to the reticulated forms, electron dense forms (possibly elementary bodies) were present in small colonies seen in some epithelial cells.

Colonies of *C. ruminantium* in the epithelial cells of salivary gland acini varied in size from 5 μm to 30 μm in diameter (Kocan *et al.,* 1987). Individual organisms within the colonies were pleomorphic with small dense staining forms in some colonies and larger individual organisms present in simple granular cells of type I and type II acini. Colonies in the salivary gland epithelial cells also contained the reticulated forms observed in the gut epithelial cells. Multiplication of the

organisms in these colonies was by binary fission (Kocan *et al.*, 1987). In the insect vector the life cycle of *C. ruminantium* is dominated by the reticulate stage (Reticulate bodies, RBs) but the elementary bodies were not noted.

### *2.4.2. Life cycle in the mammalian host.*

*Cowdria ruminantium* proliferates in the vascular endothelium, neutrophils, macrophages and reticulo-endothelial cells of mammals (Cowdry, 1925a; Prozesky and Du Plessis, 1987; Logan *et al.*, 1987b). Various stages of the life cycle have been observed by light and electron microscopy in endothelial cells of animals dying from heartwater (Pienaar, 1970; Du Plessis, 1985a). *C. ruminantium* was observed first in endothelial cells of blood capillaries of renal glomeruli and the superficial layer of the grey matter of the brain (Cowdry, 1925a).

Primary development of *C. ruminantium* is thought to occur in the reticulum of lymph nodes draining tick feeding sites. The rickettsiae may be disseminated by macrophages which phagocytose *C. ruminantium* at the site and transport them to the regional lymph node. After multiplication and maturation they are released via the efferent lymph into the blood stream where they infect endothelial cells of the blood capillaries in various organs including the brain (Du Plessis, 1970b, 1985b; Ilemobade, 1976). The life cycle in the mouse is reported to consist of two phases of rickettsaemia Du Plessis (1982). Ilemobade (1976) was of the opinion that *C. ruminantium* is introduced directly into the circulatory system of ruminants following tick feeding, is taken up by macrophages and transported to the regional lymph nodes where the first phase of replication occurs. After maturation of EBs,

infected cells rupture liberating the EBs which enter the blood stream via efferent lymph and become the first phase of rickettsaemia. The EBs in the blood stream invade and replicate in endothelial cells. After maturation the EBs are released by cell lysis into the blood to form the second rickettsaemia.

The life cycle of *C. ruminantium* in mammals was not clearly understood until the organism was grown successfully *in vitro* in cell cultures of endothelial cells and neutrophils (Bezuidenhout, Camilla and Barnard, 1985; Bezuidenhout, 1987a; Logan *et al.,* 1987b; Jongejan *et al.,* 1991c). Growth of *C. ruminantium* in neutrophils is characterised by the appearance of one to several small cocci, large forms or rods located in membrane bound phagosomes undergoing binary fission.(Logan *et al.,* 1987a).

The life cycle of *C. ruminantium* in bovine endothelial cells has been described in detail by Jongejan *et al.* (1991c). In these cells it is characterised by two forms, an extracellular infectious form the elementary body (EB) and an intracellular vegetative form the reticulate body (RB). The life cycle in the endothelial cells begins when EBs are internalised by a process similar to phagocytosis (Prozesky and Du Plessis, 1987; Jongejan *et. al.,* 1991c). The EBs become surrounded by a double membrane originating from the host cell cytoplasm and within 2 to 4 days they change to reticulate bodies of 1.2 to 2.0 μm in diameter. These RBs develop and undergo binary fission, reorganisation and condensation of DNA material, and subdivide again by binary fission. After 3 to 4 days in cultures the RBs become smaller in size with an electron dense core to become intermediate

bodies (IBs). The IBs undergo further DNA condensation without cell division to become EBs which are released into growth medium by lysis of the host cell. The complete life cycle in cultured endothelial cells takes 5 to 6 days from infection to release of EBs

## 2.5. Transmission of Heartwater.

### *2.5.1. Hosts and vectors of Cowdria ruminantium.*

Heartwater is a disease of ruminants especially cattle, sheep and goats (Alexander, 1931; Neitz, 1939; Ilemobade, 1976; Camus and Barre, 1988) and wild ruminants such as antelopes and blesbucks (Neitz, 1933, 1937). Dardiri, Logan and Mebus (1987) produced heartwater by experimental infection in white tailed deer (*Odocoileus virginianus*) and Okoh, Oyetunde and Ibu (1987) found *Cowdria ruminantium* at post mortem in brain smears from a captured Sitatunga (*Tragelaphus spekii*) infested with *A. variegatum*. Several species of wild ruminants can also be infected (reviewed by Oberem and Bezuidenhut 1987b).

### *2.5.2. Vectors.*

*Cowdria ruminantium* is transmitted naturally by ticks in the genus *Amblyomma*. Transmission occurs transtadially from larvae to nymphs and from nymphs to adults and to a minor extent transovarially (Uilenberg, 1983; Bezuidenhout and Jacobz, 1986; Camus and Barre, 1988). Thus any of the 3 stages can transmit the disease to the mammalian hosts. The most efficient vectors are the adult females whereas the males appear to be poor vectors (Alexander, 1931;

Ilemobade, 1976; Ilemobade and Leeflang, 1978). Ticks can remain infected for 15 months to 3 years (Ilemobade, 1976; Neitz, 1968 ).

Two species of *Amblyomma* are the main vectors in sub-Saharan Africa and the Caribbean islands. In Southern Africa *A. hebraeum* (Lounsbury, 1900) is the principle vector while in the rest of Africa and the Caribbean it is *A. variegatum* (Daubney, 1930b). The other species in this genus are less efficient vectors, (Walker and Olwage, 1987). Eleven species (9 African and 2 American) of *Amblyomma* can transmit heartwater (Uilenberg, 1983) including *A. gemma* and *A. lepidum* (Ngumi *et al.,* 1997). Ticks infected as larvae transmit the disease as nymphs while those infected as nymphs transmit as adult.

### *2.5.3. Distribution of Amblyomma ticks.*

The distribution of the tick vectors of *C. ruminantium* has been reviewed by Uilenberg (1983) and Camus and Barre (1988). *A. variegatum* is well adapted to parasitise domestic livestock and has a wide distribution throughout most parts of tropical sub-Saharan Africa, the islands around the east and west coast of Africa, to the south of the Arabian Peninsula and several of the Caribbean Islands. *A. hebraeum* is limited to southern and south-eastern Africa (Uilenberg, 1983; Camus and Barre, 1988). Other species of the tick which can potentially transmit *C. ruminantium* include *A. gemma* and *A. lepidum* which inhabit the dry areas of eastern and north-eastern Africa and Kenya (Ngumi *et al.,* 1997), *A. pomposum* in Angola and parts of southern and central Africa, and *A. cohaerens* which infests the savannah buffalo of central and eastern Africa and cattle in parts of Ethiopia

(Pegram, Hoogstraal, and Wassef, 1981). The main factors which determine the distribution of the vectors are height above sea level, temperature, rainfall, vegetation, seasonality and hosts. Adult *A. variegatum* are adapted to live on ruminants. Larvae and nymphs of the tick however are less discriminate and can feed on any mammal, an adaptation which enables them to survive in situations where alternative hosts are present. Once the ticks are infected they remain infective for life and transmit the organism to subsequent instars.

### *2.5.4. Artificial transmission of heartwater.*

Heartwater was first transmitted artificially to susceptible animals by inoculation of infectious blood from an infected animal (Edington, 1898; Dixon, 1898 cited by Provost and Bezuidenhout 1987). This was followed by the work of Spreull (1904) and Theiler (1905). Alexander (1931) showed that administration of infected blood by the intravenous route (i.v) is more reliable than any other route and that the size of inoculum was also critical. Alexander (1931) found that i.v administration of 10 ml or 5 ml infectious cattle or sheep blood respectively was adequate to cause disease in the two species. Theiler and Du Toit (1928) showed that *C. ruminantium* could be transmitted by i.v injection of emulsified infected *A. hebraeum* nymphs demonstrating for the first time that nymphs could act as vectors. Other organs such as mesenteric and popliteal lymph nodes from sheep infected with heartwater were used to transmit the disease to sheep (Du Plessis, 1970b) and infected brain suspensions were infective to cattle sheep and goats by i.v inoculation (Uilenberg, 1971).

Although 5 ml of infective blood stabilate has been used as the standard dose for inducing heartwater in small ruminants for many years, lower amounts of infective blood have been used. Heartwater has been induced in sheep with as little as 0.02 ml of virulent blood using the i.v route (Camus and Barre, 1988). Other workers have used 2 mls of virulent infective blood to induce hearwater in goats (Tafesse, 1992). In the bovine 5 mls of infective blood or 4 mls of infective nymph suspensions have been used (Oberem and Bezuidenhout, 1987; Lawrence *et al.* (1993).

EBs *in vitro* culture are highly infectious (Jongejan et al., 1991c). The animal infective dose using EBs and *in vitro* culture has not been thoroughly investigated but is of critical importance to these studies.

The other routes of inoculation which have been used to induce heartwater with varying degrees of success include the subcutaneous (Uilenberg, 1983; Ilemobade and Blotkamp, 1976, 1978), intracerebral, intrathoracic and intramuscular routes (Camus and Barre, 1988).

Although the source, dose, concentration and size of the infecting inoculum is important, the route of administration is critical and i.v. is the most successful, giving infection rates of 98-100%, depending on the species being inoculated (Uilenberg, 1983). Susceptibility to infection also varies among domestic ruminants, cattle are less reactive to i.v inoculation than sheep and goats (Van de Merwe, 1979; Du Plessis and Bezuidenhout, 1979).

It is clear from the foregoing review that the amount of inoculum, its origin, the route of inoculation and the susceptibility of the recipient species determines the outcome and response to experimental infection. Experimental challenge of goats and sheep in this study were inoculated by i.v since it is the most reliable and successful method of infecting ruminants.

### *2.5.5. Cultivation of C. ruminantuim.*

Cultivation of *C. ruminantium* in experimental hosts and under i*n vitro* conditions is reviewed by Camus, *et al.*, (1996).

#### *2.5.5.1. Experimental animals.*

Attempts have been made to cultivate *C. ruminantium* in various experimental animals with varying degrees of success. Animals that have been used include rats, guinea pigs (Balozet, 1935, 1936), ferrets (Mason and Alexander, 1940), albino rats deprived of vitamin $B_2$ (Hudson and Henderson, 1941), rabbits (Mason and Alexander, 1940), hamster (IEMVT, 1968) and vervet monkey (Pelissier, Troquereau and Triquier, 1950). However, it was not until Haig (1952) successfully passaged *C. ruminantium* in mice 9 times without losing infectivity for sheep that the use of mice for experimental infection became established. Ramisse and Uilenberg (1971) repeated the work of Haig (1952) successfully. The first mouse pathogenic *C. ruminantium* was isolated by Du Plessis and Kumm (1971), the Kumm stock. Since then other mouse pathogenic stocks have been isolated (Mackenzie and van Rooyen, 1981; Mackenzie and McHardy, 1984; Du Plessis, 1985a), the Kwanyanga, Nonile and Welgevonden stocks respectively. The clinical

signs of cowdriosis in the mouse appear 24 hours before death and include, increased respiratory rate, ruffled hair and difficulty in movement (Mackenzie and McHardy, 1984). The discovery of mouse pathogenic stocks of *C. ruminantium* led to the development of a mouse model for experimental studies (Du Plessis and Kumm, 1971). Since then mice have been used in experiments to predict drug activity against *C. ruminantium* (McHardy and Mackenzie, 1984, 1987a), for studies of immunity (Du Plessis *et al.,* 1991) tick infection rates (Du Plessis, 1985a), genetic resistance (Du Plessis *et al.*, 1990a) and pathogenesis (Du Plessis, 1975b).

#### *2.5.5.2. In vitro cultivation.*

Research in *Cowdria* was greatly constrained until its cultivation *in vitro* was achieved. *C. ruminantium* was first cultivated successfully *in vitro* by Bezuidenhout *et al.* (1985) in bovine endothelial cells which had been irradiated to retard their growth. It has since been cultivated in non-irradiated endothelial cells in several laboratories (Bezuidenhout, 1987a; Yunker *et al.*, 1988; Jongejan *et al.*, 1991c; Byrom and Yunker, 1990; Byrom *et al.,* 1991; Pow, Paxton and Sumption, 1993). Growth media such as Leibovits $L_{15}$ supplemented with 0.45% glucose and Glasgow Modified Eagles Essential Medium (GMEM) are used routinely to isolate and grow *C. ruminantium* (Camus, *et al.,* 1996). *In vitro* cultivation of *C. ruminantium* has led to a more complete understanding of its life cycle (Jongejan *et al.,* 1991c). It has enabled production of organisms for infection studies, identification of antigenic components, preparation of inactivated immunogens, and

isolation of pure DNA for molecular characterisation and cloning of genes coding for protective antigens for recombinant vaccine development.

### *2.6. Pathogenesis of Cowdriosis.*

The pathogenesis of cowdriosis is not clearly understood and to date no satisfactory explanation has been put forward to explain the lesions observed at postmortem (Camus and Barre, 1988). However a number of hypotheses have been advanced.

#### *2.6.1. Accumulation of fluids in body cavities.*

Accumulation of fluid in the thoracic cavity, pericardium, lung and the brain has been attributed to increased capillary permeability which leads to seepage of protein resulting in transudation of fluid through serous membranes (Clark, 1962; Uilenberg and Camus, 1993). Severe lung oedema has been attributed to the increased permeability of alveolar walls due to direct damage to the endothelial cell junctions. However, gaps in the endothelium have not been demonstrated in histological sections (Prozesky and Du Plessis, 1985a). Furthermore, cytopathic changes in the parasitised cells cannot be observed microscopically indicating that the organisms may not be responsible for the increased vascular permeability (Pienaar, 1970; Prozesky and Du Plessis, 1985a). Pulmonary oedema in turn leads to the concurrent respiratory distress observed in peracute and acute cowdriosis. Increased capillary permeability and nervous signs which occur in peracute cowdriosis have been attributed to the activity of a toxin (Jackson and Neitz, 1932; Bezuidenhout, 1982). Bezuidenhout (1982) suggested that a toxin may be the most

likely cause of the increased capillary permeability because acute pulmonary oedema occurs in the absence of large number of organisms. A toxin or immune complexes were thought to be responsible for severe renal lesions in Angora goats (Prozesky and Du Plessis and 1985c). ''Endotoxin'' activity has been demonstrated in the blood of sheep experimentally infected with heartwater (van Amstel *et al.*, 1988a; 1994). Two peaks of activity were demonstrated, one occuring shortly after the febrile reaction and the other at the time when the animal was showing severe clinical signs such as laboured breathing, cyanosis and recumbency. Van Amstel *et al.* (1988a) demonstrated that protein leakage and increased capillary permeability occurred at the onset of the febrile reaction, they concluded that the endotoxin activity was responsible for increased capillary permeability. Lipopolysaccharide has not been demonstrated in *Ehrlichia* unlike other rickettsiae (Woldehiwet and Ristic, 1993) therefore LPS may not be responsible for this effect.

Pienaar, Basson and van de Merwe (1966) suggested that brain oedema was associated with vasculitis caused by the organism in endothelial cells with subsequent destruction of the endothelium leading to focal haemorrhage and oedema. The same authors attributed glial lesions to the proximity of the neurological cells to the infected endothelium. These hypotheses have not been proved and other factors such as cytokines may be responsible for the lesions. Involvement of cytokines in the pathogenesis has not been determined but is strongly implicated.

The role of cytokines in the pathogenesis of disease in animals and man is now clearly established. In sheep infected with *C. ruminantium,* Interleukin-6 (IL-6) induces secretion of acute phase proteins by hepatocytes and this cytokine is involved in the inflammatory process of heartwater (Bensaid, Bourdulous, Lerhun *et al.*, 1993). These authors suggested that prolonged high levels of IL-6 provoke uncontrolled inflammatory reactions which lead to the lesions observed in heartwater. IL-6 is produced by macrophages and endothelial cells and acts as a promoter of immune responses. It upregulates immunoglobulin (Ig) production and is also involved in T cell activation, growth and differentiation (Shizuo, Tetsuya, and Tadamitsu, 1993). IL-6 stimulates hepatocytes to produce numerous antiproteases which negatively regulate the inflammatory response. In humans and rodents there is a feedback loop in which IL-6 inhibits lipopolysacchide or tumor necrosis factor (TFN-α) induced IL-1 production (Schindler *et al.,* 1990). Another pro-inflammatory cytokine produced by macrophages which has synergistic interactions with TNF-α during the inflammatory process is IL-1 (Cavallion, 1993). They both activate endothelial cells and induce production of IL-6 and IL-8. IL-1 is a potent endogenous pyrogen and together with TNF-α and IL-6 stimulates fever. IL-6 and TNF-α produce fever at higher concentrations than IL-1 and in humans IL-1 induces fever, slow wave sleep (dullness) hemodynamic shock, leukopenia and hypotension (Dinarello *et al.,* 1993, cited by Delannoy, Leukeux and Miossec 1993). TNF-α in high doses leads to hemodynamic, pulmonary metabolic and pathological consequences indistinguishable from endotoxaemia and septic shock.

It further induces vascular permeability oedema and injury to endothelial cells and appears to play a key role in systemic hypotension and altered metabolism. TNF-α has been incriminated in lung injury in sheep (Redl, Schlag and Lamche, 1990), cattle (Kenison, Elsasser and Fayer, 1991; Gerros, *et al.*, 1993) and goats (van Miert, van Duin and Wensing, 1992). The role played by cytokines in the pathogenesis of heartwater is unfolding slowly and will become clearer as research efforts are now being focused in this area.

Death in heartwater is attributed to extreme pulmonary cardiac distension or pulmonary oedema (Uilenberg, 1977; Abdul-Rahim and Shomein, 1978), to suppression of gammaglobulin response (Ilemobade, 1976) or to circulatory collapse (Clark, 1962; Owen *et al.*, 1973). Circulatory collapse could be attributed to the effects of IL-1 and TNF-α as both are capable of inducing hemodynamic shock and hypotension (Delannoy, Lekeux and Miossec, 1993). Extensive nephritis which leads to irreversible kidney damage has been proposed as another cause of death in heartwater (Prozesky and Du Plessis, 1985a). Another possibility is asphyxia caused by extensive pulmonary oedema which is observed consistently in peracute heartwater. Despite the many proposed causes of death in heartwater, no firm consensus has been established. Death due to asphyxia resulting from pulmonary oedema seems to be the most plausible explanation although goats and sheep do not always show severe pulmonary oedema.

### *2.6.2. Clinical signs of heartwater in ruminants.*

The clinical signs of heartwater have been reviewed by Camus and Barre (1988), Camus *et al.*, (1996), Uilenberg (1983) and van Pypekamp and Prozesky (1987). The clinical disease occurs in three main forms: peracute, acute and inapparent (Alexander 1931; Neitz, 1968). The symptoms observed in heartwater are not characteristic and the form of disease which develops depends on the species, immune status, age, individual susceptibility, and the stock of *C. ruminantium* causing the infection.

The incubation period of the natural disease varies between 7 and 35 days with an average of 14-18 days (Alexander, 1931; Neitz, 1968; Uilenberg, 1983). The incubation period of experimental infections on the other hand varies from 5 to 14 days depending on the infecting dose, the type of inoculum and the route of inoculation. In sheep, it takes 5 to 9 days while in cattle it takes 11 to 12 days (Alexander, 1931; Neitz, 1968; Uilenberg, 1983) or shorter (van de Merwe, 1979).

Small ruminants are more susceptible to heartwater than cattle. They develop peracute, acute and mild forms of this infection while cattle are more resistant.

**Peracute heartwater.**

The peracute disease is common in exotic breeds and is accompanied by sudden death with or without clinical symptoms in some animals. Animals which survive longer develop fever in excess of 41°C followed by prostration, collapse, convulsions, accelerated respiratory and pulse rates followed by death within 36

hours (Uilenberg, 1983). A small proportion of sheep and goats show anorexia, dullness, nystagmus, bleating, forced respiration, frequent urination and defaecation, chewing movements and lateral recumbency followed by leg-pedalling (Karrar, 1966; Spruell, 1922).

**Acute form.**

The acute form of heartwater is frequently seen in sheep and goats. It may occur with or without pronounced symptoms after development of fever. This form lasts 2 to 6 days. It is accompanied by a rapid temperature rise to 41 or 42 °C which persists throughout the infection. The main clinical signs include: progressive unsteady gait, holding the head down, listlessness, lateral recumbency, increased respiratory and pulse rates, paddling and continuous galloping, licking of the lips and nystagmus (Alexander, 1931) and occasionally diarrhoea (Uilenberg, 1981). The disease can also occur as a subacute form in which less pronounced symptoms develop over 7 to 10 days or as a chronic or inapparent form which is accompanied by transient fever (Camus and Barre, 1988).

The disease in cattle is characterised by similar but less severe clinical signs to those observed in sheep and goats.

The mortality rates of heartwater in ruminants range between 5 and 100% and are dependent on the same conditions which affect the development of the clinical disease (Camus and Barre, 1982; Haig, 1955; Uilenberg, 1981).

## 2.7 *Post Mortem (p.m.) Changes Observed in Cases of Cowdriosis in Domestic Ruminants.*

### *2.7.1. Macroscopic pathology.*

The lesions which occur in heartwater infection of domestic ruminants are always similar, but variable in extent and not pathognomonic (Uilenberg, 1981; Prozesky, 1987b). The pathology of heartwater has been reviewed by Uilenberg (1981) and Prozesky (1987b). In the following review the major and striking changes which have been reported are given on a system to system basis. The peracute cases normally show the severest lesions.

### *2.7 2. Respiratory system.*

The most consistent pathological change in the respiratory system observed in natural and experimental heartwater is marked oedema of the lungs. This oedema can be diffuse with widened interlobular septae (Prozesky and Du Plessis, 1985c). Hydrothorax is also present with accumulation of a variable amount of clear yellow fluid ranging from 40 ml in sheep and goats to 60ml or more in cattle. Lung oedema is severest in animals which die from the peracute form of the disease (van de PypeKamp and Prozesky, 1987). On cut surfaces, the lungs ooze a serous and frothy fluid, the trachea and bronchi are filled with froth and the mucosae are often congested and contain serofibrinous exudate.

### *2.7.3. Circulatory system.*

Pronounced hydropericardium is commonly seen on necropsy examination. This lesion is usually more severe in small ruminants than in cattle (Henning, 1956;

Uilenberg, 1981; Prozesky, 1987b). Other changes include haemorrhages on the heart muscle, and oedema of perirenal and mediastinal tissue (Uilenberg, 1981).

### *2.7.4. Lymph nodes.*

The lymph nodes are usually enlarged and oedematous especially the mediastinal and bronchial lymph nodes (Uilenberg, 1981; Prozesky and Du Plessis, 1985c; Prozesky, 1987b). On the cut surface, some of these lymph nodes especially the retropharyngeal, submaxillary, cervical, bronchial and mediastinal have petechial haemorrhages (Alexander, 1931).

### *2.7.5. Spleen.*

Splenomegaly may or may not be present and is considered an infrequent lesion in heartwater (Uilenberg, 1981; Andreasen, 1974; Ilemobade, 1976). This lesion, however, has been observed in many cases examined in South Africa (Prozesky, 1987b).

### *2.7.6. Urinogenital system.*

In some reported cases of heartwater the kidneys are swollen, slightly pale in sheep and congested in cattle (Steck, 1928 cited by Prozesky, 1987b). Oedema of perirenal tissues has been reported in a few cases. The kidneys of experimentally infected Angora goats were markedly enlarged, pale and with moderate oedema of perirenal tissues (Prozesky and Du Plessis, 1985c).

### *2.7.7. Brain.*

Oedema of the brain may or may not be present at p.m. (Uilenberg, 1983). When it is present, the gyri of the cerebellum may be swollen and when the oedema

is severe partial prolapse of the cerebellum has been observed. Other lesions include congestion of the meninges, with accumulation of excessive fluid in the subarachnoid space, thickening of the choroid plexus, petechiae, and ecchymoses in the mid brain, brain stem and cerebellum (Pienaar, Basson and van de Merwe, 1966).

### *2.7.8. Other organs.*

Congestion and oedema of the mucosa of abomasum, accompanied with occasional petechial haemorrhages is a regular lesion in cattle but rare in sheep and goats (Uilenberg, 1981). Petechiae have also been reported in the mucous membranes of the urinary bladder, vagina and conjunctiva and haemorrhages have been observed in the rectum accompanied by enterophagia (Prozesky, 1987b). Hepatic lesions are uncommon in this disease, although slight to mild hepatomegaly has been reported in some cases. However the gall bladder is normally distended (Uilenberg, 1981; Prozesky, 1987b).

### *2.7.9. Microscopic pathology.*

The most consistent finding in all animals is the presence of large numbers of *C. ruminantium* colonies in endothelial cells of alveolar capillaries and the brain. The other lesions are not characteristic. The only microscopic changes observed in parasitised endothelial cells is distension (Cowdry, 1925a), or occasional occlusion of the capillary lumens ( Prozesky and Du Plessis, 1985c).

### *2.8. Immunity to Cowdriosis.*

Specific immunity develops in animals which recover from heartwater infection (Stewart, 1987). Recovered animals have solid immunity to homologous challenge and show some degree of resistance to heterologous challenge (Neitz, 1939; Neitz and Alexander, 1941; Stewart, 1987; Du Plessis *et al.*, 1989). This immunity lasts for a variable period of time in different species. In sheep which recover from experimental infection it lasts for 7 to 34 months extending possibly to 48 months (Neitz, Alexander and Adelaar 1947) while in cattle it lasts between 4 months and 3.5 years (Neitz and Alexander, 1945; Chabeuf, 1976; Arnold and Asselberg, 1981). The duration of protective immunity in ruminants seems to be associated with premmunity since a carrier status has been established in animals which recover from heartwater. (Ilemobade, 1976; Andrew and Norval, 1989b; Camus, 1992).

#### *2.8.1. Innate resistance and breed susceptibility.*

Young animals are resistant to cowdriosis for the first few weeks of life (Neitz and Alexander, 1941). Lambs and calves up to 3 weeks old and kids up to 6 weeks of age are relatively resistant to heartwater (Neitz and Alexander, 1941; Thomas and Mansvelt, 1957). A few young animals, however, succumb to infection at these ages (Uilenberg, 1971; Du Plessis and Malan, 1988). Creole goat kids are resistant until they are two weeks old (Camus, unpublished data, cited by Uilenberg and Camus, 1993) the exception being Angora goats which are highly susceptible (Du Plessis, Jansen and Prozesky, 1983). Susceptibility in older

animals, depends on previous exposure to the disease and breed. Uilenberg (1983) found no differences in susceptibility between Zebu and Taurine breeds in non endemic areas. In endemic areas local zebu and breeds such as the N'Dama and the Bauole are very resistant (Camus and Barre, 1990). In contrast, breeds such as the Sahiwal and the Brahman are as susceptible as Taurine breeds. African Zebu from cowdriosis free areas are also as susceptible to heartwater as the Taurine breeds when introduced to *Amblyomma*-infested areas (Gueye Mbengue, Kebe and Diouf., 1982; Logan, Tembley and Miller, 1988). In terms of species resistance, cattle are more resistant to cowdriosis than sheep and goats (Du Plessis and van Gas, 1989). Among the small ruminants Merino sheep were found to be more susceptible than Persian sheep (Alexander, 1931). Alexander (1931) noted in South Africa that indigenous sheep were more resistant than Merinos but goats were as susceptible as the Merinos, he observed that in general animals raised under endemic conditions were more resistant than those that were not.

### *2.8.2. Humoral immunity.*

Animals which recover from natural and experimental heartwater infections develop specific antibodies to *C. ruminantium* (Camus and Barre, 1987; Du Plessis and Malan, 1987a; Semu, Mahan, Yunker and Burridge, 1992). Three serological tests are used frequently to monitor antibody responses to *C. ruminantium,* they are: Indirect flourescent antibody test (IFAT), Enzyme linked immunosorbent assay (ELISA), and Western blotting. IFAT using mouse or endothelial cell derived antigens (Du Plessis, 1982; Brown, Logan, *et al.*, 1989; Du Plessis, *et al.,* 1993;

Lawrence, *et al.*, 1993), indirect ELISA using soluble antigens (Soldan *et al.,* 1993; Neitz *et al.*, 1987) and competitive ELISA (Jongejan *et al.*, 1991a; Du Plessis *et al.*, 1993) have been used. None of these tests however has acceptable specificity however, ELISA using a recombinant fragment of the Major antigenic protein-1 (MAP-1) (van Vliet *et al.*, 1995) has improved specificity. This recombinant polypeptide (MAP-1b) does not react with *Ehrlichia ovina* although it reacts with *E. chaffeensis* and *E. canis* sera. Western blotting has been used to detect antibodies to immunogenic proteins of the EB (Jongejan and Thielemans, 1989a; Rossouw, *et al.,* 1990; Mahan *et al.*, 1993).

Circulating specific antibodies to *C ruminantium* were detected by direct ELISA in serum samples from goats immunised with inactivated elementary body antigens on day 10 post inoculation (Tafesse, 1992). After immunisation of mice with elementary bodies, antibodies were detectable in serum IFAT by day 14 post inoculation (Byrom, Mahan and Barbet, 1993).

The role of antibodies in immunity to heartwater is not clear, as conflicting reports are found in the literature. Experimental transfer of serum or gamma globulin did not confer protection and *in vivo* neutralisation tests have given variable results (Du Plessis, 1970a, 1993; Uilenberg, 1983). Du Plessis, Bezuidenhout and Ludeman (1984) found no correlation between antibody titre and immunity to heartwater in calves. Sera from 10 calves of 12 whose blood was not infective to sheep were positive by IFAT 3 months after immunisation, while 17 of 18 calves whose blood was infective were also serologically positive by IFAT. The

same authors found no correlation between replication of *C. ruminantium* in calves with a high degree of natural resistance and their serological responses and eventual immunity to challenge. Du Plessis, *et al.,* (1989) compared 10 stocks of *C. ruminantium* for cross immunity, serological responses and mouse pathogenicity. They found that the antigenic diversity of the stocks could not be correlated with antibody levels detected by IFAT since sera raised against all the 10 stocks reacted positively to Kumm stock antigen which showed no cross-protection. Furthermore variation in titres could not be related to the stock of *C. ruminantium* used. Similarly Martinez *et al.* (1993) observed that sera from animals which had survived challenge after immunisation did not neutralise *C. ruminantium* infection of endothelial cell cultures *in vitro.*

In contrast to the above results Du Plessis and van Gas (1989) found positive correlation between antibody responses of naturally infected sheep and goats and their susceptibility to experimental challenge. All seronegative animals developed the disease when challenged while seropositive animals resisted challenge. In cattle, however, large numbers of seronegative animals have been found to be partially or totally resistant to natural challenge (Camus and Barre, 1988; Du Plessis and Malan; 1987a). Protection of these animals could be due to cell mediated immunity which these authors did not determine. Byrom, Mahan and Barbet (1993) showed that hyperimmune serum from mice inhibited adhesion and entry of *C. ruminantium* to and into cultured endothelial cells. They also demonstrated that two bovine sera one each from experimental and natural

infection, inhibited *C. ruminantium* from infecting endothelial cell cultures. Byrom Mahan and Barbet (1993) however observed that passive transfer of antibodies given in serum or as gamma globulins given simultaneously with infection, during incubation or during clinical reaction did not confer protection to susceptible mice. Furthermore, transfer of immune serum alone or with complement failed to protect mice against intravenous challenge with *C. ruminantium* (Byrom, 1993, unpublished data). In contrast Du Plessis (1993) found that homologous serum in the presence of complement inhibited the infectivity of *C. ruminantium* (Kumm stock) to outbred mice. In three experiments, 2 out of 5, 1 out of 5 and none out of 5 mice died following pre-treatment of *C. ruminantium* with increasing amounts of complement while 4 out of 5 mice died in the absence of complement. The mechanisms by which antibody affects the infectivity of *C. ruminantium* in the presence of complement may be by antibody dependent complement mediated lysis or directly by activation of the alternate complement pathway leading to lysis ( Du Plessis, 1993).

The role played by antibodies in immunity to other rickettsial species is better established; antibodies are protective in many cases (Tringali *et al.*, 1983). Transfer of immune serum confers protection on challenged animals. Antibodies enhanced opsonisation and destruction of rickettsiae by neutrophils and macrophages (Gambril and Wisseman, 1973) *in vitro*. Experimental studies with immune sera indicated that macrophages destroy *Rickettsia mooseri* in the presence of antibodies but not in their absence (Gambril and Wisseman, 1973; Beaman and

Wisseman, 1976). Furthermore, there is good correlation between antibody titres, macrophage activation and levels of immunity (Kekcheeva *et al.,* 1981). The presence of immune sera enhances the activity of macrophages, indicating the complementarity of humoral and the cell-mediated immune responses.

It has been shown experimentally that antibodies restrict the infectiveness of *Coxiella burnetti* and enhance its uptake by macrophages and granulocytes (Abinanti and Marmion, 1957; Kazar, Skultetyova and Brezina, 1975). Kishimoto and Walker (1976) found that immune serum rendered *C. burnetti* more susceptible to phagocytosis by guinea pig peritoneal macrophages. Lewis *et al.* (1978) found that addition of immune serum greatly reduced the multiplication of *Ehrlichia canis* in macrophage cultures. Passive transfer of immune serum from mice which had recovered from *E. risticii* infection prevented the disease in 22 out of 24 recipients, moreover, purified immunoglobulin G (IgG) from immune serum protected mice from infection with *E. risticii* while IgG from non immune serum did not provide protection (Kaylor *et al.,* 1991). Passive transfer of serum enhanced clearance of rickettsiae in animals experimentally infected with *R. mooseri* and *R. tsutsugamushi* indicating that there was cross protection among closely related organisms (Shirai *et al.,* 1976).

It is evident from the above review that antibodies can enhance destruction of rickettsiae by macrophages and that passive transfer of antiserum provides immunity in some rickettsial species with similar life cycles and *Coxiellae*. This role which has not been established in heartwater demands thorough investigation.

### *2.8.3. Cellular immunity.*

*Cowdria ruminantium* is an intracellular rickettsia which parasitises the endothelial cells of blood capillaries of ruminants (Cowdry, 1925a; Prozesky and Du Plessis, 1987a; Jongejan *et al.,* 1991c), neutrophils, (Logan *et al.*, 1987b); macrophages and monocytes (Du Plessis, 1970b; Ilemobade, 1976; Pienaar, 1970). The intracellular location of *C. ruminantium* during part of its life cycle confers a degree of protection from antibody responses. However, intracellular parasites are subject to attack by the parasitised cells and by cell mediated immune responses with effector mechanisms such as cytotoxic T cells and natural killer (NK) cells.

Cell-mediated immune mechanisms have evolved to control intracellular infections. These responses are regulated by T lymphocytes which control the immune response by organizing other effector cells (Kaufmann, 1988). T lymphocytes represent the major mediators and coordinators of acquired resistance against intracellular bacteria (Hahn and Kaufmann, 1981). T cells are broadly divided into 3 types based on their antigen receptor type and functionally. $CD4^+$ (T helper/Th) cells, $CD8^+$ (cytotoxic/TC/CTL) cells and $CD4^-CD8^-$ or γδ T cells (Mombaerts *et al.*, 1993). $CD4^+$ T lymphocytes recognise antigen presented in association with self molecules of class II Major Histocompatibility Complex (MHC class II) present on antigen presenting cells such as macrophages, dendritic cells and other cells of the reticuloendothelial system. $CD8^+$ T cells recognise antigen in association with self MHC class I molecules present in all nucleated cells. Unlike antibodies which recognise antigens directly, T cells recognise non-

conformational fragments of processed antigens which are usually peptides of 8-20 amino acids. The peptide fragments recognised by T cells are processed in endosomal or cytoplasmic compartments. They are attached to MHC class II or class I molecules, transported to the cell membrane and presented to $CD4^+$ or $CD8^+$ T cells respectively (Harding and Song, 1994). The antigen receptors on $CD4^+$ and $CD8^+$ are heterodimeric structures consisting of alpha and beta polypeptide ($\alpha\beta$) chains. The $CD4^-CD8^-$ T cells have receptors composed of $\gamma\delta$ polypeptide chains. These receptors are termed T cell receptors (TCRs). In addition to TCRs, $CD4^+$ and $CD8^+$ T cells also express CD3, a molecule which is intimately associated with TCR and is involved in initiation of cell activation after antigen stimulation (Fitch, 1986). The $\gamma\delta$ cells recognise antigen by a different pathway which is not yet completely understood.

$CD4^+$ T cells in mouse and man are composed of two subsets, defined by the profiles of cytokines they secrete, as Th1 and Th2 type (Mossman and Coffman, 1989). The Th1 and Th2 T cells control both the humoral and cell-mediated responses by producing cytokines which cross-regulate each other. Resting T cells produce only IL-2, but after stimulation they produce other cytokines. The initial activation step starts after antigen presentation. Th1 cells produce interferon gamma (IFN-$\gamma$), interleukin-2 (IL-2) and tumour necrosis factor beta (TNF-$\beta$) and their effect is to promote cell mediated immunity and support $IgG_2$ antibody production. Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 which support $IgG_1$, IgE and IgA antibody responses and eosinophilia. Cytokines such as IL-3, tumour

necrosis factor alpha (TNF-α) and granulocyte macrophage colony stimulating factor (GM-CSF) are secreted by both Th1 and Th2 cells (Kemeny, Noble, Homes and Diaz-Sanchez, 1994).

$CD8^+$ T cells are also classified into two distinct subsets based on the cytokines they secrete. Type I $CD8^+$ T cells secrete the same cytokines as Th1 T cells and are restricted by MHC class I. Type II $CD8^+$ T cells secrete Th2 type cytokines and are restricted by MHC class II (Kemeny *et al.*, 1994). These subdivisions of $CD8^+$ have been observed *in vitro* and also *in vivo* as observed in T cell clones derived from patients with lepromatous leprosy (Kemeny *et al.,* 1994). The three types of T cells present in peripheral blood mononuclear cells (PBMC) of humans and mice are found in the peripheral blood of ruminants. In mature ruminants the majority of T cells are $CD4^+$ representing 25 to 35% of PBMC while $CD8^+$ constitute 15 to 25%. $CD8^+$ T cells mediate MHC class I restricted cytolytic T cell activity against virus-infected cells and those infected with protozoa and other intracellular bacteria (Howard and Morrison, 1994). The third T cell type in ruminants are the gamma delta (γδ) T cells. These γδ T cells form a major proportion of T cells in the peripheral blood of young ruminants. The mean proportion of γδ T cells in calves 1 to 3 weeks of age ranges between 27% to 60%. In mature ruminants this cell type constitutes 5 to 10% of PBMC (Hein and Mackay, 1991). The cytokine profile of the γδ T cells is similar to that of Th1 cells and they are cytotoxic against allogeneic cells (Mackay *et al.*,1988). The role played by γδ T cells in immunity in the young is thought to be that of providing an

early cover of a non-MHC restricted cellular immunity until a mature $CD4^+$, $CD8^+$ ($\alpha\beta$) T cell system becomes established (Hein and Mackay, 1991).

Cellular immunity in hearwater infections in the mouse is mediated by $CD8^+$ T cytotoxic cells (Du Plessis *et al.*, 1991). Adoptive transfer of $CD8^+$ (Lyt-$2^+$) from mice immune to *C. ruminantium* to non-immune recipients conferred protection to lethal challenge. In addition, adoptive transfer of immune cells depleted of Lyt-$2^+$ ($CD8^+$) cells were unable to confer protection against challenge whereas depletion of $CD4^+$ ($L_3T_4^+$) T cells had no effect on protection (Du Plessis, Berche and Van Gas, 1991). Flow cytometric analysis of purified lymphocytes obtained from specificied pathogen free BALB/c mice infected with *C. ruminantium* and treated to prevent death provided further evidence that $CD8^+$ T cells are protective (Du Plessis, Gray and Van Strijp, 1992). These authors showed that there was significant increase in the population of Lyt-$2^+$ T cells after challenge and that the population of $L_3T_4^+$ dropped below pre-infection levels except on day 34 after challenge. The role played by $CD8^+$ T cells in protective immunity in ruminants against Cowdriosis is unknown and this requires to be determined.

### *2.8.4. The role of cytokines in cell mediated immunity.*

Cytokines play a key role in influencing the type of immunity which develops in the animal. In addition some cytokines have a direct effect on the invading organism. Interferon gamma (IFN-$\gamma$) and alpha (IFN-$\alpha$) have anti-viral activity *in vitro* and *in vivo* (Tizard, 1994). These cytokines are also known to interfere with rickettsia (Byrne and Turco, 1988; Manor and Sarov, 1990), to be inhibitory to

chlamydia (Shemer and Sarov, 1988) and to exhibit anti-*Cowdria* activity *in vitro* (Totte *et al.*, 1993; Mutunga and Sumption, 1995; Mahan *et al.*, 1996). *In vitro* experiments have demonstrated that IFN-γ and IFN-α have anti-rickettsial effects that result in clearance of organisms from the cytoplasm of some infected cells (Turco and Winkler, 1983a, 1993; Wisseman and Waddel, 1983; Jerrels, Turco, Winkler and Spitalny, 1986; Turco and Winkler, 1983b ; Turco and Winkler, 1988). Inteferons are important mediators of anti-rickettsial activity. TNF-α is an mediator in the clearance of *R. conorii* from murine fibroblasts through induction of nitric oxide synthesis (Feng and Walker, 1994). Recombinant bovine gamma interferon (rBOIFN-γ) reduces the *in vitro* growth of the Senegal, Welgevonden and Gardel stocks of *C. ruminantium* from bovine endothelial cells. It also inhibits growth of *C. ruminantium* in caprine jugular endothelial cells (Totte, Blankaert, Zilimwabagabo and Werrenne, 1993). The same authors found that cattle which resisted experimental infection of heartwater produced significantly higher levels of circulating IFN-α than those which did not resist challenge indicating that IFN-α may be partly responsible for resistance shown by the cattle which survived challenge. However multiple effectors are likely to be responsible for protective immunity to *C. ruminantium.*

### *2.9. Control of Cowdriosis.*

Heartwater is controlled by three main methods: vector control, chemotherapy and vaccination.

### *2.9.1. Vector control.*

Vector control of heartwater has been reviewed by Norval, (1991). It is achieved by using acaricides. This was first reported by Dixon (1899) and Alexander (1931) and is aimed at interrupting the life cycle of the vector (Camus *et al.*, 1996) by application of acaricides. Two main methods of application termed as intensive and strategic or seasonal control are used. Eradication of *Amblyomma* ticks is extremely difficult and only limited success has been achieved (Howel, *et al.*, 1981).

Intensive tick control is aimed at controlling all stages of ticks throughout the year in order to keep the tick numbers to a minimum. Its advantage is that of reducing production losses due to tick worry and tick-borne diseases. This method is recommended for farms with highly susceptible animals such as dairy breeds and in marginal areas where *Amblyomma* species are found occasionally or where ecological conditions are unsuitable for long-term survival of the ticks (Bezuidenhout *et al.*, 1994). Two disadvantages associated with this method are: the fact that total tick control is a considerable cost and loss of immunity to tick-borne diseases owing to lack of tick challenge (Bezuidenhout, 1987b). In addition, chemical control of heartwater in the absence of other tick-borne diseases is not justifiable (Pegram and Chizyuka, 1990).

Strategic or seasonal control of adult ticks during the rainy season or when tick numbers pass a limit is more economical. This type of control is aimed at reducing the number of vectors in order to promote a stable disease situation and

limit the adverse effects of tick worry. The advantages of this method are lower dipping costs and the acquisition of immunity against heartwater by animals, leading to an epidemiologically stable situation. Despite these advantages, implementation of this method is complicated by the presence of other tick borne diseases and this makes it difficult to formulate one policy of strategic control for all of them (Bezuidenhout and Bigalke, 1987).

Acaricides are applied in two ways, spraying and dipping. Spraying is achieved by hand pump when few animals are being treated or by spray races for large numbers of animals. This method has the disadvantage that animals are not normally covered fully with the acaricide. Plunge dipping is the best method of applying acaricide since it gives a complete coverage of the body as cattle are fully immersed (FAO, 1984; Young, Groocock and Kariuki, 1987). Dip concentrations, however, require to be tested regularly to determine the replenishment rate to maintain active concentration, or they can become a source of acaricide resistance when their management is not strict. When dipping is done correctly effective control of heartwater can be achieved (Currasson, 1932; Norval, 1979). Use of acaricides to control tick vectors has been practised in eastern and southern Africa since the turn of this century and has limited the establishment of *Amblyomma* ticks and heartwater in the highveld of Zimbabwe (Norval and Lawrence, 1979). It has also achieved localised eradication in some parts of South Africa (Stampa, 1969) and Kenya. Despite this success, use of acaricides to control heartwater faces many problems and does not give a long-term solution. Major problems associated with

the vector control method have been reviewed by Norval *et al.*, (1991). The six most important were considered to be:

1. The cost of acaricides and their application are becoming too high in relation to income of farmers in many developing countries.
2. Organisational problems of national tick control programmes and vulnerability to political and economic instability.
3. Development of resistance to acaricides.
4. The results of intensive tick control upon endemic stability for heartwater and other tick-borne diseases.
5. That tick control has little effect on abundance of *Amblyomma* where alternate hosts for the adult stage are present because attached males emit Aggregation Attachment Pheromone (AAP) that causes unfed nymphs and adults to attach in preference to untreated hosts (Norval *et al.*, 1989a).
6. Dipping or spraying with acaricides pollutes the environment and may result in residues in milk and meat.

Alternative ways of controlling vectors of heartwater without dipping and spraying have been used successfully. Significant control of *A. variegatum* in experimental studies has been achieved by use of APP to attract unfed nymphs and adults to the source of acaricide (Norval *et al.*, 1989a, 1989b, 1991). Synthetic pheromone incorporated in acaricides can be used as a slow releasing decoys attached to the host (Norval *et al.*, 1991). The advantages of this method over traditional dipping or spraying include, the use of less acaricide per head and

therefore lower costs; it is more environmentally friendly, longer lasting and reduces the need for frequent mustering of animals for dipping or spraying, control is specifically toward *Amblyomma* ticks and does not affect the epidemiology of other tick-borne diseases. The use of APP to attract unfed ticks leads to effective control of *A. variegatum* and *A. hebraeum* even in the presence of alternate hosts (Norval *et al.*, 1991).

### *2.9.2. Chemotherapy.*

Sulfonamides and tetracyclines have been used to treat heartwater successfully (Camus *et al.*, 1996). Other chemotherapeutic agents have also been used with variable success (van Amstel and Oberem, 1987).

The first drug used successfully to treat heartwater was Uleron, a short acting sulfonamide (Neitz, 1940). Treatment during the early stage of the reaction gave greater success. Later, Alexander, Neitz and Adelaar (1946) successfully used Uleron and sulphapyridine in treating natural cases of heartwater. Gloxazone has also been used to treat clinical cases of heartwater (Synge and Scott, 1976; Du Plessis, 1981). The toxicity of gloxazone however made it unsuitable for treating heartwater (Camus and Barre, 1982). A range of tetracyclines, chlortetracycline aureomycin, oxytetracycline, rolitetracycline and doxcycline have been used extensively to treat heartwater (van Amstel and Oberem, 1987). Successful therapy depends on the time the treatment is given, the dose of chemotherapeutic agent used, the formulation and the route of administration (van Amstel and Oberem, 1987). Treatment during the incubation period alters the subsequent course of the

disease completely, resulting in complete blocking of the clinical response or leads only to a temporary febrile response without any symptoms developing; The curative dosage varies from 2 mg/kg to 40 mg/kg body weight (kg/bwt) dose (Weiss *et al.*, 1952; Haig *et al.*, 1954). It is given as a single dose or two divided doses over a 24 hour period. A single dose of 20 mg/kg bwt given during the early febrile stage is curative but in advanced stages larger and multiple doses become necessary. As to which formulation is the most effective, Uilenberg (1971) did not find any advantage using different formulations. Various routes of administration have been used to treat heartwater. Karrar and Elhag-Ali (1965) used the oral route to treat sheep and cattle with minimum therapeutic doses of 8 mg/kg bwt for sheep and 500 mg/kg bwt for cattle given daily until recovery. The most common and most effective routes are the intramuscular and intravenous (Camus and Barre, 1988).

Other antibiotics such as streptomycin have been used with limited success ranging from no effect (Synge and Scott, 1976) to moderate effects (Du Plessis *et al.*, 1983). Despite the successes achieved with vector control and chemotherapy, the limitations which reduce the effectiveness of these two methods demand that animals be protected against heartwater by immunisation.

### *2.9.3. Control by vaccination (immunisation)*

#### *2.9.3.1. History.*

The first reported attempt to protect animals against heartwater was by Dixon (1898) (cited by Camus and Barre, 1988). Dixon used a mixture of bile and

infected blood or bile mixed with pericardial fluid to inoculate animals. The trial produced no conclusive results except that young animals seemed to have been protected. This was possibly due to innate resistance rather than the vaccination since young animals are known to be relatively resistant to heartwater in the first few weeks of life. Theiler (1904) used polyvalent serum, produced by combining serum from sheep and goats which had been previously infected and presumably recovered, to protect goats against experimental infection with some limited success. The first proper vaccine trials against heartwater were attempted by Hutcheon (1902), Spreull (1904) and Theiler (1905, 1906) (cited by Oberem and Bezuidenhout, 1987a).

Three findings are considered of historical importance in the development of a feasible method for vaccination against heartwater. These are: 1. Alexander (1931) showed that i.v. inoculation of infected blood was the most successful way of infecting ruminants, 2. Neitz (1940) reported that heartwater could be treated with the sulphonamide, uleron, and 3. Neitz and Alexander (1941) showed that young animals were resistant to heartwater. These findings led to the immunisation of resistant young animals by infection and treatment method (I/T) using live virulent vaccines. Two other methods of immunisation using attenuated and inactivated organisms have been used to protect ruminannts against this infection. The three methods of of immunisation are reviewed.

### *2.9.3.2. Live vaccines.*

Immunisation for protective immunity against heartwater can be induced in susceptible ruminants by infection with virulent blood or with supernatant from ground-up ticks which were previously allowed to feed on infected animals followed by treatment of the animal with oxytetracycline at the beginning of the febrile reaction to cowdriosis (Jongejan, *et al.*, (1993). Animals which recover from heartwater are immune to homologous challenge (Oberem and Bezuidenhout, 1987a). Two live virulent vaccines, the infected blood and infected tick vaccines, (FAO, 1984; Bezuidenhout, 1981; Oberem and Bezuidnhout, 1987b) have been tested extensively and found to protect domestic ruminants against heartwater. The infected blood vaccine commercially produced in South Africa is obtained from sheep experimentally infected with the Ball 3 reference stock (Bezuidenhout *et al* 1994). The infected tick vaccine is a supernatant of a ground-up tick suspension from tick nymphs or larvae infected with the Ball 3 stock (Bezuidenhout, 1981). Both vaccines are administered intravenously at pre-determined concentrations to infect animals, the animals are allowed to develop a temperature reaction and treated once or twice on the second or third day of fever with oxytetracyline at 20 mg/kg bwt. A second treatment is given if the fever continues. Recovered animals are solidly immune to homologous challenge (Uilenberg, 1983). The effectiveness of the blood vaccine is greater than 95% (FAO, 1984). Live vaccine have been used successfully for many years to protect animals against heartwater in South

Africa where up to 250,000 doses are being used annually (Bezuidenhout *et al.*, 1994).

Despite the success of the live vaccines they are limited by the following disadvantages: they are laborious and expensive to produce, they are labile requiring storage under liquid nitrogen to maintain their efficacy, their administration through the i.v. route usually requires qualified personnel, there is also a possibility of transmitting other tick-borne diseases, or the development of adverse reactions in the young especially following administration of tick suspensions (Oberem and Bezuidenhout, 1987a), and the danger of virulence of the vaccine. Another serious limitation with this type of vaccine is the existence of distinct antigenic differences between *C. ruminantium* stocks (Du Plessis and Van Gas, 1989; Du Plessis *et al.*, 1989; Jongejan *et al.*, 1991b) which restricts its use to certain areas only. Furthermore the live vaccine is not recommended for pregnant animals (FAO, 1984). However, one major advantage with the live vaccine is that only one immunisation in the lifetime of the animal appears to be required in the endemic areas where natural challenge occurs (FAO , 1984).

#### *2.9.3.3. Attenuated live vaccines.*

The limitations associated with live virulent vaccines led to the search for alternative types of vaccines. Live attenuated vaccines against heartwater became possible following the successful cultivation of *C. ruminantium* in tissue culture (Bezuidenhout, *et al.,* 1985). Sequential passage of the Senegalese and the Gardel isolates in bovine umbilical endothelial cells resulted in attenuation of the organism

(Jongejan, 1991; Jongejan, *et al.*, 1993b). Vaccination of sheep with the attenuated organisms protected them against lethal challenge with the virulent homologous isolate. A solid protective immunity was developed by the immunised animals. Responses to heterologous challenge were variable depending on the isolate. For instance immunised animals were not protected against the Welgevonden stock (Jongejan, 1991). A trial with another cell culture, attenuated Senegalese isolate vaccine had moderate success under field conditions (Gueye, Jongejan, Mbengue and Uilenberg, 1994). In this trial 30 sheep were immunised and 30 served as controls. Upon natural challenge 22 sheep in the control group died from heartwater while 8 of the vaccinated died from cowdriosis and 5 died from inter-current infections. These results indicate that attenuated vaccines may be useful in controlling heartwater in the future. The fact that only two stocks of *C. ruminantium* have been attenuated successfully limits their use due to limited cross-protection between many stocks of this organism. Attenuated vaccines are advantageous in that natural infection is simulated to a much higher degree than is possible with inactivated vaccines and the animal should acquire a long-lasting cell-mediated and humoral immunity. They are also practical and economical since they may only be administered once in the life-time of the animal. The major risks with this type of vaccine are reversion to virulence by the organism if it is not attenuated sufficiently and possible transmission of the low virulence strains. Recent studies in Kenya (Ngumi 1997) show that low virulence isolates do not protect against challenge with highly virulent isolates but highly virulent isolates

protect against a wide range of stocks. Furthermore animals immunised with low virulence isolates succumbed more quickly on challenge with virulent stocks. This suggests that attenuation may increase susceptibility on challenge.

#### *2.9.3.4. Inactivated vaccines.*

Inactivated vaccines have gained importance in the 1990s and reports of their use are increasing. Martinez *et al.* (1993, 1994) immunised 9 goats with inactivated elementary bodies of the Gardel stock of *C. ruminantium* suspended in Freund's complete adjuvant. Each goat received 2 subcutaneous injections. Challenge of the vaccinated animals with a lethal dose of homologous isolate caused temperature reaction in 6 out of 9 animals and 3 died. All unvaccinated goats died within 7 to 12 days after challenge. Tafesse (1992) immunised 12 goats and 10 mice with inactivated elementary bodies of the Welgevonden isolate in Freund's complete adjuvant using two regimens. Six goats received primay inoculation only and the other 6 received primary and booster inoculations 21 days apart. Upon challenge with the virulent Welgevonden stock a total of 7 out of 12 (3 given primary immunisation only and 4 given two doses) were protected against lethal homologous challenge all controls were not. Surprisingly, immunisation of mice with inactivated elementary bodies did not confer protective immunity upon challenge with virulent homologous stock but survival rates was increased.

Mahan, Andrew, Tebele, Burridge and Barbet (1995) reported that they had successfully protected sheep against heartwater after immunisation with inactivated *C. ruminantium* from Zimbabwe emulsified in Freund's complete adjuvant. In their

trial they used three groups of 5 sheep. Two groups were immunised with inactivated organisms, one group received intact organisms without adjuvant, the second group received the organism in Freund's complete adjuvant for primary inoculation the third group was used as control. Subsequent booster inoculations of immunised groups were delivered in Freund's incomplete advujant. Animals which received a primary dose of inactivated EBs in FCA and a booster in FIA were protected against homologous challenge while the others were not, indicating immunisation induced protective immunity. The future of inactivated vaccines against heartwater is promising.

Inactivated vaccines have several advantages over virulent and attenuated live vaccines. These advantages include: No risk of reverting to virulence, the ease administration therefore no requirement for qualified personnel, thermostability, no risk of transmitting other diseases to the vaccinated animals and no export of virulent stocks of *C. ruminantium* to other countries where they do not exist. Despite the fact that inactivated EBs induce protective immunity in a high proportion of immunised animals, they are still limited by the low level of cross-protection among *C. ruminantium* stocks. This requires that other types of vaccines be developed such as subunit, recombinant or nucleic acid vaccines which may overcome the problem of limited cross-protection observed with the existing vaccines.

### *2.9.3.5. Recombinant vaccines.*

Recombinant DNA technology has led to the isolation of genes encoding important immunogenic antigens of pathogenic organisms and to the production of measurable quantities of antigens. Some recombinant antigens have been used successfully to protect animals against homologous and heterologous challenge. Genes for the 32kDa Major antigenic protein 1 (MAP1), (van Vliet. *et. al.*, 1994), 21kDa MAP2 protein (Mahan *et al.*, 1994b) and 58kDa heat shock protein (Hsp) (Lally *et al.,* 1995) of *C. ruminantium* have been cloned and the antigens expressed in *E. coli*. These recombinant antigens are currently being tested for their immunogenicity. It is anticipated that these antigens may become candidates for vaccines for controlling heartwater in the future. One advantage of recombinant DNA technology is that it is possible to identify the gene of a conserved protective antigen and to clone it.

Recombinant antigens of a number of rickettsial organisms have been used successfully to protect animals against lethal homologous challenge. Viswanath, MacDonald and Watkins (1990) cloned and expressed a 5.5 kilobase Hind III fragment (gene encoding 198kDa protein) from *Rickettsia conori* Kenya tick typhus genomic DNA into *E. coli* JM107. Immunisation of guinea pigs with sonicated lysates of this recombinant *E. coli* strain expressing the 198kDa protein led to the development of antibodies which recognised the native 198kDa protein. The immunised animals were protected from experimental infection with the homologous *Rickettsia.conorii* species and were also partially protected from

experimental infection with the heterologous species *Rickettsia rickettsii*. MacDonald, Maclean, Mann and Milch (1988) cloned and expressed a gene encoding a 155kDa protein of *R. rickettsii* in *E. coli* JM107. Immunisation of guinea pigs with sonic lysates of the recombinant JM107 [pGAM22] expressing this protein protected the animals from experimental infection with *R. rickettsii*. In other experiments, immunisation with lysates of *E. coli* containing pUC8 subclones of a 155kDa recombinant protein protected mice upon lethal challenge with *R. rickettsii* (MacDonald Anacker and Garjian, 1987). Recombinant antigens have also been used experimentally as vaccines to protect animals against ehrlichial infections. An affinity purified 55kDa recombinant protein was used to protect mice against lethal challenge of *E. risticii* (Dutta, Shankarapa and Matingly-Napier, 1991). Shankarapa, Dutta and Martingly-Napier (1991) used a 44kDa recombinant protein of *E. risticii* to protect mice against lethal challenge with the homologous strain. They also demonstrated potentiation of protective immunity by immunising mice with two recombinant antigens of 44kDa and 70kDa proteins. Recombinant antigens of other intracellular pathogens such as *Mycobacteria* have also been used to immunise and protect experimental animals against lethal homologous challenge. Silva and Lowrie (1994) successfully vaccinated mice with recombinant heat shock protein Hsp65 of *Mycobacterium leprae* to protect them against the homologous organism and *M. bovis* (BCG) and *M. tuberculosis* H37RV. The vaccine used was composed of monocyte-like murine tumour cells transfected with a retroviral shuttle plasmid [pZIPNeoSV(X)] expressing the gene for the *Mycobacteria* Hsp. Silva *et*

*al.* (1994) demonstrated that antigen specific T cell clones from the spleens of immune animals were capable of transferring immunity to non vaccinated recipients. The cell types responsible for this transfer were $CD8^+$ T cells and were shown to effectively and specifically lyse mycobacteria infected macrophages. The above method is a form of DNA immunisation using antigen presenting cells to process genes expressed in APC.

This brief review, indicates that recombinant antigens are becoming increasingly important as candidate vaccines for controlling infectious diseases. It is hoped that on going research on recombinant antigens of *C. ruminantium* will lead to identification of an antigen to fulfil this role.

The immune mechanisms responsible for protection against heartwater in the ruminant have not been fully determined. Therefore, in the following work I sought to attempt to characterise the mechanisms responsible for protective immunity against the Welgevonden stock of *C. ruminantium* by:

1. Characterisation of surface exposed antigenic proteins of the elementary body (infective stage) of *C. ruminantium* (Chapter 4).
2. Characterisation of Isotype-specific IgG responses of ruminants and mice immunised with live, inactivated and recombinant antigens of this stock (Chapter 5).
3. Investigating the cellular immune responses to immunisation with live or inactivated antigens of *C. ruminantium* before and after challenge of immunised and control goats (Chapter 6).
4. Investigating the immune responses and level of protection resulting from immunisation with a recombinant heat shock protein (GroEL) of *C. ruminantium*. This represents the first report of a trial of recombinant antigens to protect against cowdriosis (Chapter 7).

# CHAPTER THREE

# GENERAL MATERIALS AND METHODS

This section describes materials and methods which are used in at least two chapters or those which could not be included in specific results chapters of the thesis.

## *3.1. Experimental Animals.*

### *3.1.1. Mice.*

Female outbred 'TO' white mice (Tuck and Sons, England) weighing 20g to 30g were used in immunisation and challenge experiments. They were kept in groups of five per cage and supplied with feed and water *ad libitum*. Mouse experiments were carried out in the small animal unit of the Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh.

### *3.1.2. Goats.*

Six mature male and female British cross-breed Saanen goats aged between 18 and 24 months were obtained from Marsh Holdings Damhead, Scotland. They were housed indoors in groups of two and supplied with water and concentrates *ad libitum*. Before being used for immunisation and challenge experiments, they were kept under quarantine, screened for the exretion of Salmonella spp. (a routine precaution for all animals brought to CTVM) and dosed with anti-helmintics. Experimental infections were undertaken in the isolation unit, CTVM.

### *3.1.3. Sheep.*

Twelve sheep were immunised with recombinant antigens. Six were 6 months old Merino crosses and the other six were 12 to 18 months old Scottish Blackface crosses obtained respectively from Blythe Bank and South Bank Farms, Peebleshire, Scotland. Before and during experiments they were treated in the same way as goats in Section 3.1.2.

## *3.2. Preparation of Peripheral Blood Mononuclear Cells (PBMC) from immunised and control animals.*

Blood was obtained from goats and sheep by bleeding from the jugular vein into 10 ml vacutainer tubes containing the anticoagulant lithium heparin (Becton Dickinson, UK). Blood was transferred into 25 ml sterile disposable universal tubes (Sterilin, UK) and centrifuged at 2,880 x g for 20 minutes at 4°C using a Megafuge 3.0 R (Heraeus Sepatech, UK). The buffy coat was taken from the interface between the red blood cells and plasma using a Pasteur pipette and mixed with 9 ml of phosphate buffered saline (PBS) containing 10 units of heparin per ml. This cell suspension was layered carefully onto 8 ml of cold Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 850 x g for 35 minutes at 15°C. The Peripheral blood mononuclear cells (PBMC) were obtained from the Ficol-PBS junction using a 5 ml pipette and mixed with 20 ml of wash buffer (PBS + 10 units/ml heparin) and washed by centrifugation at 450 x g for 10 minutes at 15°C. The PBMC pellet was re-suspended in fresh 20 ml wash buffer and washed again. Washed PBMC was re-suspended in 2-3 ml of complete RPMI 1640 medium containing 8% goat serum (collected from CTVM) or

foetal bovine serum (Life Technologies, Paisley, Scotland). Viability and cell concentration was determined by vital staining with 0.45% trypan blue and counting of unstained cells using a Neubauer chamber. Cell concentration was adjusted to 2 x $10^6$ cells/ml in complete RPMI 1640 medium for cell proliferation assays or flow cytometric analysis of lymphocyte subsets.

### *3.2.1. Depletion of adherent cells from PBMC to obtain effectors for cytotoxic assays.*

Peripheral blood was obtained from sheep before and after immunisation and PBMC was prepared as described earlier (Section 3.2). Then a suspension of PBMC in RPMI-1640 medium, was added into 25 ml tissue culture flasks (Nunc, Inter Med, UK) and incubated for 2 hours at 37°C in 5% $CO_2$ to remove adherent cells. Non-adherent cells were washed out of the flask with warm medium and sedimented at 450xg for 10 minutes at 15°C (Megafuge 3.0, Heraeus Sepatech, UK). The pellet was suspended in 2 ml RPMI 1640 medium. Viability was determined by vital staining with 0.45% trypan blue in PBS and the cells counted and their numbers adjusted to 1.0x$10^6$ cells/ml. The 'pure' lymphocytes were used as effectors in cytotoxic assays (section 7.2.3.5).

## *3.3. Preparation of Serum.*

Blood samples were collected into 10 ml vacutainer tubes without anticoagulant from the jugular veins of goats and sheep. Seven millilitres of blood from sheep or goats was taken. Blood was obtained from the tail veins of mice using a 25 gauge needle and at least 0.1 ml was withdrawn after warming the mice for 15

minutes by placing their cage on top of a radiator until the veins were prominent. The blood samples were held at 37°C for 1 hour, adherent blood clots were loosened and the samples stored at 4°C overnight. Serum was aspirated into centrifuge tubes and centrifuged at 2650 x g for 30 minutes at 4°C and aliquoted into 1 0 ml Eppendorf or 5 ml plastic bijoux bottles and stored at -20°C until use .

### *3.4. Growth of C. ruminantium in vitro and preparation of Elementary Bodies (EBs) for experimental use.*

Elementary bodies (EBs) of *C. ruminantium* were the source of antigens for the cell proliferation assays, Western blotting and enzyme linked immunosorbent assays (ELISA).

Three stocks of *C. ruminantium* were propagated in two bovine endothelial cell lines derived from bovine aortic cells/bovine aortic endothelial cells (BAC/BAE) or bovine pulmonary cells (BPC). Uninfected cultures were maintained in Glasgow Minimum Essential Medium (GMEM/BHK21), (Life Technologies, Paisley, Scotland) supplemented with 20% inactivated, foetal bovine serum, 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml Amphotericin B (Antibiotic/Antimycotic) in 25, 75 and 175 $cm^2$ plastic tissue culture flasks (Nunc). Cells were grown to near confluency (6 to 8 hours) and infected with *C. ruminantium* elementary bodies (EBs) from a supernatant of an infected bovine endothelial cell culture. Briefly: Five millilitres and 7.5 ml of infective culture medium were used to infect 25 $cm^2$ or 75 $cm^2$ flasks, and 175 $cm^2$ flasks respectively. The Infected cultures were maintained in GMEM containing 10% foetal bovine serum, 10% tryptose phosphate broth, 2mM L-glutamine, 20mM Hepes buffer and

antibiotic/antimycotic. Cultures were incubated at 37°C in 5% $CO_2$ for 10 to 14 days and the EBs harvested. The EBs were harvested by removing half the culture medium and replacing it with fresh medium. The withdrawn culture medium was placed in 10 ml centrifuge tubes, centrifuged at 450 x g for 5 minutes to remove cell debris. The supernatants were centrifuged at 15,000 x g for 20 minutes at 4°C in a Varifuge 20RS (Heraeus Sepatech UK) to pellet the EBs. The supernatant was discarded and the pellet of EB's was washed twice by re-suspending them in 20 ml of sterile PBS followed by centrifugation at 15,000 x g for 15 minutes then the supernatants were discarded. The washed EBs were stored in 50-100 µl of PBS in 1.5 ml Eppendorf tubes and kept at -20°C.

### *3.5. Plasmid vectors and bacterial strain used for recombinant antigens.*

A 7.4kb fragment of *C. ruminantium* DNA containing the GroEL operon was identified by immunoscreening a genomic DNA library (Lally *et al.*, 1995). *In vivo* excision of the insert in pBluescript II (Strategene) permitted growth in the *E. coli* (JM109) host. A 0.7 kb HindIII fragment from GroEL was subcloned into pTrcHis (Invitrogen) by Dr. Susan Nicol of the CTVM and grown in XL1 blue cells (Stratagene) for expression and purification of a 35kDa product (pTrcHis 0.7).

### *3.6. Growth of E. coli JM109/pBScript, JM109/pBSCr9.4, XL1-blue/pTrcHis, and XL1-blue/pTrcHisHIII0.7 (pTrcHis0.7).*

Cultures of *E. coli* JM109/pBScript, JM109/pBSCr9.4, XL1-blue/pTrcHis, XL1-blue/pTrcHisHIII0.7 were grown by plating glycerol stock cultures on LB agar and incubating overnight at 37°C. Single colonies were picked, inoculated into 10 ml LB broth and grown at 37°C overnight with vigorous shaking. These cultures

were used to inoculate 500 ml of LB broth medium and cultures were incubated to log phase where OD600 was between 0.6 and 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1mM final concentration to cultures of XL1-blue/pTrcHis and XL1-blue/pTrcHis 0.7 and incubated further at 37°C for 4 hours followed by centrifugation at 4000 x g for 15 minutes using an MSE centrifuge to collect the cells. Then the pellets were kept at -20°C until needed.

Bacterial antigens were prepared after 100 µl of each bacterial pellet were dissolved with equal amounts of 2X SDS sample buffer and subjected to SDS-PAGE using 10% acrylamide as described in Section 3.5. Determination of the presence of 58kDa recombinant antigen was carried out by Western blot analysis as described in Section 4.2.4. The membranes were screened with a 1/50 dilution in PBST of day 28 serum from an infection and treatment (I/T) goat (G69). After ascertaining the presence of the recombinant antigens, bacterial pellets were inactivated by suspending them in 0.15% formalin then incubating for 30 minutes at room temperature washed twice in sterile PBS and lysed on ice using an a sonicator. Protein estimation was carried out as described in Section 3.10 then, the concentration of each antigen was adjusted to 2 mg/ml with PBS. Antigens were prepared for inoculation by mixing equal volumes of each antigen with either Complete Freund's adjuvant (FCA, Sigma, UK), Incomplete Freund's adjuvant, (FIA, Sigma, UK) or Mantonide ISA50 (Seppadec'Quay d'Orsay, Paris, France).

### *3.7. Preparation and purification of 35kDa recombinant protein (subclone of GroEL) of C. ruminantium from E. coli lysate on nickel columns.*

The DNA insert (0.7kbHIII fragment) was positioned downstream and in frame with the sequence that encodes an N-terminal polyhistidine fusion peptide (Lally *et al.*, 1995). The polyhistidine residues binding domain of the peptide allows for a single step purification of recombinant protein. The Histag protein binds to the column with greater affinity so that any host proteins which bind non-specifically to the resin could be easily washed away followed by elution of the bound protein. The nucleotide and amino-acid sequence of GroEL showing the Hind III restriction sites are given in Figure 3.1. Growth of *E. coli* (XL1-blue/pTrcHis0.7) was carried out as described in Section 3.6.

The Xpress ™ Protein Purification system (Invitrogen) for Histag proteins was carried out following the manufacturer's instructions but without satisfactory results, and an alternate purification system was optimised for use.

The Qia-express Ni-NTA (Qiagen) protein purification system was modified and used to purify the protein as follows: the bacterial pellet was removed from -20°C, thawed for 15 minutes at room temperature, resuspended in 1.0 ml buffer B (8M Urea, 0.1M $NaOH_2PO_4$, 0.01M Tris/HCl, pH 8.0), mixed vigorously and allowed to incubate for 1 hour at room temperature with constant mixing. The sample was sonicated on ice using a MSE sonicator (MSE, UK). Cellular debris were removed by centrifuging the lysate at 10,000 x g for 10 minutes at room temperature and the supernatant was collected.

Small quantities of the 35kDa subclone (Histag) protein were purified using Ni-NTA spin columns following the manufacturer's instructions: Six hundred millilitres of the cleared lysate were loaded onto a pre-equilibrated Ni-NTA spin column and centrifuged for 2 minutes at 2000 rpm. The flow through was collected and analysed by SDS-PAGE to check for binding. The spin column was washed twice with 600 µl of wash buffer (8M Urea, 0.1M Na-phosphate, 0.01M Tris/HCl, pH 6.5; 32mM imidazole (buffer C) by centrifugation at 2000 rpm for 2 minutes each. Protein was eluted with 2 x 200 µl buffer of 8M Urea, 0.1M Na-phosphate, 0.01M Tris/HCl, pH 4.5 (Elution buffer/E) by centrifugation at 2000 rpm for 2 minutes each. The eluate was collected into 1.5 ml Eppendorfs and 10 µl aliqouts were analysed by SDS-PAGE on 10% acrylamide gels, and stained with Coomassie as described in Section 3.10. A light box was used to locate protein bands and ascertain the purity of the recombinant protein. The gels were photographed as described in Section 3.11.

Larger quantities of the 35kDa subclone (Histag) protein were purified using 5 ml columns by gravity. Five millilitres of mixed Ni-NTA resin was allowed to settle in the column by gravity and the preservative (20% ethanol) was drained out to the level of the resin. Seven millilitres (three column volumes) of 8M Urea, 0.1M $NaOH_2PO_4$, 0.01M Tris/HCl, pH 8.0 (buffer B) was passed through the column to wash out the ethanol then 10 ml of cleared bacterial lysate was passed through the column at a flow rate of 10 ml per hour. The column was then treated with 10 column volumes (25 ml) of binding buffer (8M Urea, 0.1M $NaOH_2PO_4$, 0.01M

Tris/HCl, pH 8.0, 0.5 %Triton X-100) at a flow rate of 10 ml per hour followed by 6 column volumes of wash buffer (8M Urea, 0.1M $NaOH_2PO_4$, 0.01M Tris/HCl, pH 6.3, 32mM imidazole, 0.5% Triton X-100).

After washing, the recombinant protein was eluted with 6 column volumes (15 ml) of elution buffer (8M Urea, 0.1M $NaOH_2PO_4$, 0.01M Tris/HCl, pH 4.5, 0.5% TritonX-100). One millilitre samples were collected and 10 µl aliqouts were analysed for protein by SDS-PAGE as described in Section 3.8.

Samples containing only the 35kDa protein were pooled together and dialysed overnight at 4°C against a buffer containing 1M urea, 0.05M.Tris/HCl, 0.005% Triton X-100, pH 8.0 using a cellulose tubing (Sigma) with a 12kDa cut off point. Further desalting was performed using Nap 10™ (Sephadex G25 columns, Pharmacia, Sweden) following the manufacturer's instructions as described in Section 3.8. The pooled dialysed desalted fractions were concentrated by freeze drying or by placing them in cellulose tubing and dipping the tube in polyethylene glycol (PEG) for 3 to 5 minutes and stored at -20°C. Purity of the protein was ascertained by Western blotting. The purified protein was used for ELISA (Section 7.2.3.5), animal inoculation tests (Section 7.2.1.4) and for cell proliferation assay (Section 6.2.4).

### *3.8. Fractionation of proteins of C. ruminantium and E. coli by Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).*

Electrophoresis was carried out according to the discontinuous bufffer system of Laemmli (1970). Large and minigels were electrophoresed in XL Protean II and

Miniprotean II Dual slab Cell apparatus (BioRad) respectively following the manufacturer's instructions using a power pack model 1000/500, (BioRad).

Stock solutions of acrylamide were mixed to obtain 4%, 10% and 12% polyacrylamide gels (Appendix A 6). Ten percent gels were used to separate *E. coli* proteins, while 12 % gels were used for separating *C. ruminantium* antigens. Stacking gels contained 4% acrylamide. Gels were cast with a single tooth preparative comb or multiple teeth comb depending on the purposes for the separation. The concentration of EB antigens loaded per gel depended on the purpose for which the antigen would be used. Samples of EBs for Western blotting were loaded at 0.272 μg/cm$^2$ or higher. Minigels were run at a constant voltage of 200v for 45 minutes. Prestained molecular weight markers (BioRad) were run alongside in each gel in 10 μl amounts.

### *3.9. Electrophoretic Transfer of C. ruminantium and E. coli Proteins onto Nitro-cellulose Membranes.*

Electroblotting of proteins was carried out as described by Towbin, Staehelin and Gordon (1979).

Following electrophoresis the gels were soaked in Bjerrum and Schaffer-Nielsen transfer buffer (48mM Tris, 39mM Glycine, 1.3mM SDS, 20% methanol) for 15 minutes. The proteins were transferred onto nitro-cellulose membranes with pore size of 0.45 μm (Hybond C fortified nitro-cellulose paper, Amersham International, UK) using a Transblot SD (Semi-dry) Electrophoretic Cell (BioRad). Electrophoretic transfer of proteins from the large gels was carried out for 45 minutes at a constant power of 10v/0.8A per gel while transfer from minigels was carried out for 30 minutes

at 10v/0.28A. After the transfer, the nitro-cellulose membranes were rinsed once and washed twice (5 minutes/wash) in PBS containing 0.1% Tween 20 (PBST) on a rocking platform. Non-specific binding was blocked by incubation of memmbranes in 5% skimmed milk (Marvel) in PBST overnight or for 1 hour on a rocking platform. Blots were then washed three times in PBST and dried by placing them between two sheets of blotting papers. The part of the membrane containing pre-stained standards was cut off and kept then the remaining part of membrane was cut into vertical strips 5 mm wide with the help of a ruler, strips were numbered sequentially and used for Western blotting.

### *3.10. Coomassie Blue Staining of Protein in SDS-PAGE Gels.*

After electrophoresis, gels were placed directly into a clean container with Coomassie stain (0.1% Brilliant blue, 40% methanol, 10% acetic acid) and left for 1 hour on a rocking platform. The staining solution was poured off and replaced with a destaining solution (40% methanol, 10% acetic acid 50%). The destaining lasted for 1 hour. Gels were examined over a light box and destained further overnight if necessary to remove background staining. The stained gels were soaked in distilled water and photographed.

### *3.11. Photography.*

Western blots and Coomassie blue stained gels were photographed with a Polaroid MP4 Land Camera (Polaroid Corp., USA) using Type 55 Polaroid film and appropriate filters.

### *3.12. Desalting of Protein Samples.*

The samples were desalted using Nap 10 columns (Pharmacia) following the manufacturer's instructions. Briefly, the top was removed, excess fluid poured off, the column supported and the gel equilibrated with 1.5 ml of deionised distilled water. The equilibration buffer was allowed to enter the gel bed and 1.0 ml of the sample was added and allowed to enter the gel bed. Protein was eluted with 1.5 ml of deionised distilled water, collected into 1.5 ml Eppendorf tubes and stored at -20°C or freeze dried overnight. Freeze dried samples were reconstituted in 50-100 µl of double distilled water before analysis by SDS-PAGE and Western blotting.

### *3.13. Determination of protein concentration of antigens (Protein Assay).*

Assays for protein concentration were carried out using the Bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford IL., USA) employing the microtitre plate protocol according to manufacturer's instructions.

Briefly: Protein standards were prepared by diluting bovine serum albumin (BSA) in distilled water to obtain a range of 200-2000 µg/ml. Ten microlitres of the standards or samples and 200µl of the BCA reagent were incubated together at 37°C for 30 minutes using a microtitre plate. The optical density (OD) at 570 nm was read using a microtitre plate reader (Multiscan Plus, Dynatech) and protein concentration was determined by reading off the concentration from a standard graph obtained by plotting the optical density values of the standards on the Y axis and their protein concentration on the X axis or by using the following following formula:

$$P = \frac{A}{B} \times C$$

where P = total protein, A = optical density of the standards with optical density closest to that of the test sample, B = optical density of the test sample and C = the protein concentration of the standard in micrograms /ml.

### *3.14. Preparation of inactivated EBs for immunisation of goats and mice.*

The Welgevonden stock of *C. ruminantium* was propagated in bovine arterial endothelial cells and the EBs were prepared as described in section 3.4. Washed EBs were re-suspended in 0.15% formalin in PBS and incubated at room temperature for 30 minutes with constant mixing. Inactivated EBs (IEBs) were pelleted at 13,000 x g (Micro Centaur, MSE, UK) then re-suspended in PBS and washed twice (5 minutes each) by centrifugation. Washed IEB's were re-suspended in 1.0 ml PBS and protein estimation was carried out as described in Section 3.13, the protein concentration adjusted to 2 mg/ml by addition of PBS. Equal volumes of IEB suspension and adjuvant were mixed using a double-hubbed method (Hebert, 1979) in which two syringes are joined by a double hubbed needle. One syringe contained Freund's complete adjuvant (FCA) and the other an equal volume of the IEB suspension in PBS. The antigen solution was passed between the syringes until a complete water-in-oil emulsion was formed. A small volume formed a discrete drop in cold water.

For booster inoculations IEBs were incorporated in Freund's incomplete adjuvant (FIA).

#### *3.14.1. Immunisation of the goats and mice with inactivated EBs.*

Two goats (G107 and G108), were inoculated intramuscularly (I/M) with 150 μg/dose of EB protein as a primary inoculation suspended in FCA. Twenty one

days later they received a booster inoculaton 75 μg/dose of IEB protein suspended in Freund's incomplete adjuvant (FIA) subcutaneously in the neck region. These goats received 5 further booster inoculations of 75 μg/dose at 5, 10, 17, 26 and 31 months post-inoculation (PI) prepared in FIA. Mice were immunised with 100 μg/dose IEB protein prepared in FCA and 50 μg/ml prepared in FIA subcutaneously on primary and booster immunisation respectively. Booster immunisation was given at day 21 PI.

### *3.14.2. Sample collection.*

Serum samples were taken from the goats on day 0, 14, 21 and day 28 post immunisation as decribed in Section 3.3.

Peripheral blood mononuclear cells were collected on day 28, day 48 and day 143 post immunisation and used for cell proliferation assays with EBs and purified components of *C. ruminantium* as stimulants. Serum for serology was collected from the mice as described earlier on days 0, 14, 21, and day 34 PI.

## *3.15. Staining of live elementary bodies with 6-Carboxyfluorescein Diacetate (6-CFDA) and their counting for use in challenge experiments.*

Freshly prepared elementary bodies of the Welgevonden stock of *C. ruminantium* were stained with 6-CFDA and counted using a Nebauer chamber and used for challenge inoculations of experimental animals.

This was achieved as follows:

1. Ninety mililitres of EBs from a highly yielding (10-14 day old) culture of the Welgevonden stock was harvested and suspended in 90 μl of complete RPMI 1640.

2. Ten microlitres of 1/10 dilution of CFDA (obtained by diluting 10 μl stock 6-CFDA in 490 μl PBS) was added immediately to the 90 μl of freshly prepared EB

suspension and the mixture was incubated in the dark at room temperature for 15 minutes.

3. The EB/6-CFDA suspension was centrifuged at 12,000 x g at 4°C, the pellet resuspended in 500 µl of PBS mixed well using a 26G needle and pelleted by centrifugation at 12,000 x g for 5 minutes.

4. The clean pellet was re-suspended in 90 µl of PBS, mixed thoroughly by vortexing. Ten microlitres of the suspension was dropped onto a microscope slide then a 22x22 mm coverslip was placed over the drop.

5. The slide was examined using a Laborflour fluorescence microscope (Leitz) fitted with an I2/3 filter block with a blue excitation filter (BP450-490) at X400 total magnification. Live cells appeared blue. The number of live cells in 10 squares of a graduated eyepiece were counted then the mean was determined to obtain the grid counts. The total number of live EBs per ml of culture was calculated by multiplication of the mean number of EBs (grid count), by the total area of coverslip (484 $mm^2$), multiplied by the area of the grid (100 $mm^2$), by the dilution factor ($X10^1$) and the sum was multiplied by 100 or by using the formula:

Total number of EBs/ml = Mean Grid count x $A_1$. x $A_2$. x Dilution factor ($10^1$) x 100.

Where $A_1$ = Area of coverslip ($mm^2$) , $A_2$ = Area of the slide ($mm^2$).

### *3.16. Challenge of animals with live virulent EB cultures of the Welgevonden stock of C. ruminantium.*

Sheep and goats were challenged in the isolation unit of the CTVM by intravenous inoculation with 2 ml virulent culture of *C. ruminantium* containing 3 x

$10^5$ EBs through the jugular vein. Mice were challenged by intravenous inoculation through the tail vein with 0.1 ml virulent culture of *C. ruminantium* containing 1 x $10^3$ EBs in the Small animal unit of the CTVM. The EBs used were counted as described earlier (Section 3.14.).

### *3.17. Isolation of Endothelial Cells from Peripheral Blood Mononuclear Cells of Sheep.*

Isolation of endothelial cells from PBMC of sheep by a method described by Albert Bensaid (Personal Communication, 1995) as follows:

1. An attempt was made to isolate endothelial cell from peripheral blood of six sheep (S66, S69, S71, S72, 74 and S76). Blood was collected in heparinised vacutainer tubes as described in Section 3.2 before the sheep were immunised with recombinant 58kDa Hsp protein of *C. ruminantium.*
2. PBMC were prepared essentially as described in Section 3.2 except that equal volumes of blood and sterile heparinised PBS were mixed, layered over ficol- hypaque and centrifuged at 1000 x g for 30 minutes at 15°C. The PBMC were removed and transferred into 10 ml RPMI 1640 medium supplemented with 8% foetal bovine serum (Life Technologies, Paisley, Scotland).
3. Cell concentration was adjusted to $10^7$ cells/ml and the same volume was added into 25 $cm^2$ or 80 $cm^2$ tissue culture flasks (Nunc, Denmark). The flasks were incubated at 37°C in a moist atmosphere with 5% $CO_2$ for 2 hours. Unattached cells were removed by washing the flasks twice with warm complete RPMI 1640 medium.

4. Adherent cells were grown in Glasgow minimum essential medium (GMEM) supplemented with 20% foetal bovine serum containing 300 μg/ml of endothelial cell growth supplement (Sigma) for 5 days at 37° in a moist chamber containing 5%$CO_2$ Half the media in the flasks containing endothelial cells was replaced, and incubation was continued until the cells were confluent then passaged. Flasks without endothelial cells were treated in the same way, incubated for 3 weeks then discarded if there was no cell growth. To passage confluent monolayers of each cell line the cells were washed once with sterile PBS, incubated in 0.1 solution of trypsin in versene at 37°C for 5 minutes and centrifuged at 1500 rpm for 15 minutes at room temperature The Cells were re-suspended in fresh medium and passaged. Cell lines were stored by re-suspending the cells in complete GMEM containing 10% DMSO and freezing at -70°C or in liquid nitrogen as described by Pow *et al.* (1993). Endothelial cells were isolated successfully from 3 of 6 sheep (S66, S69 and S72) and used as autologous targets in cytotoxicity assays (Section 7.2.3.5).

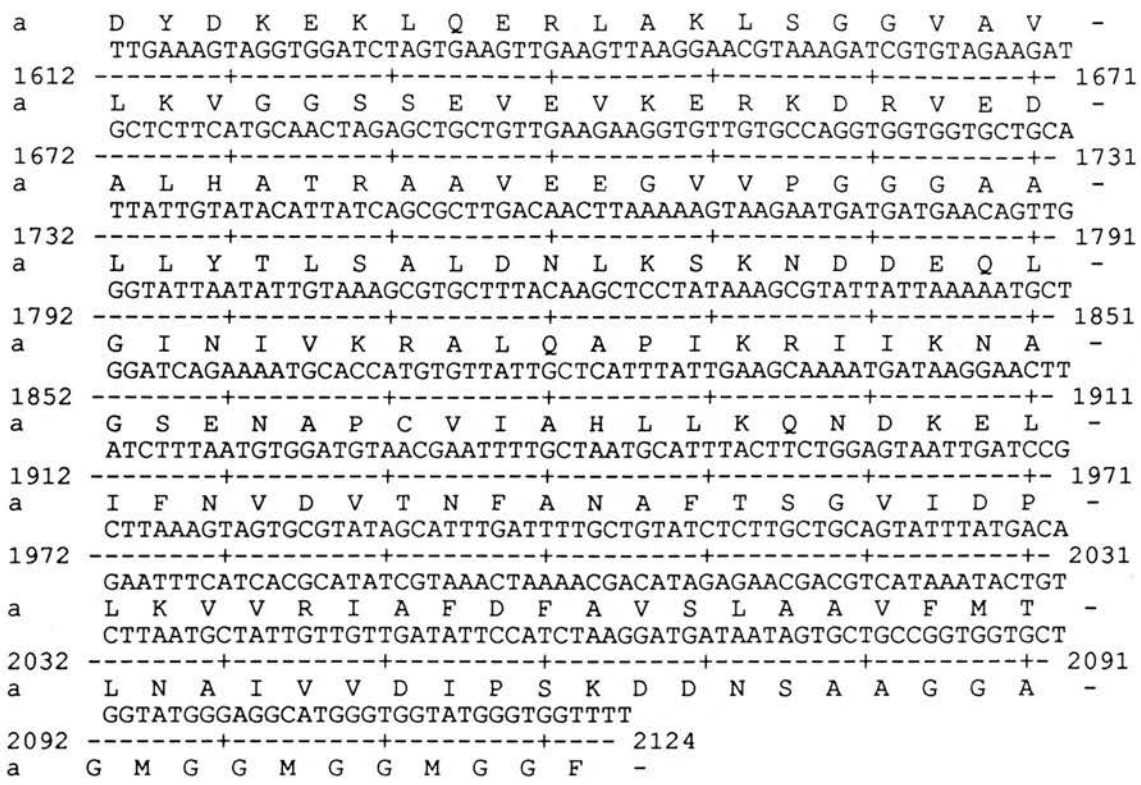
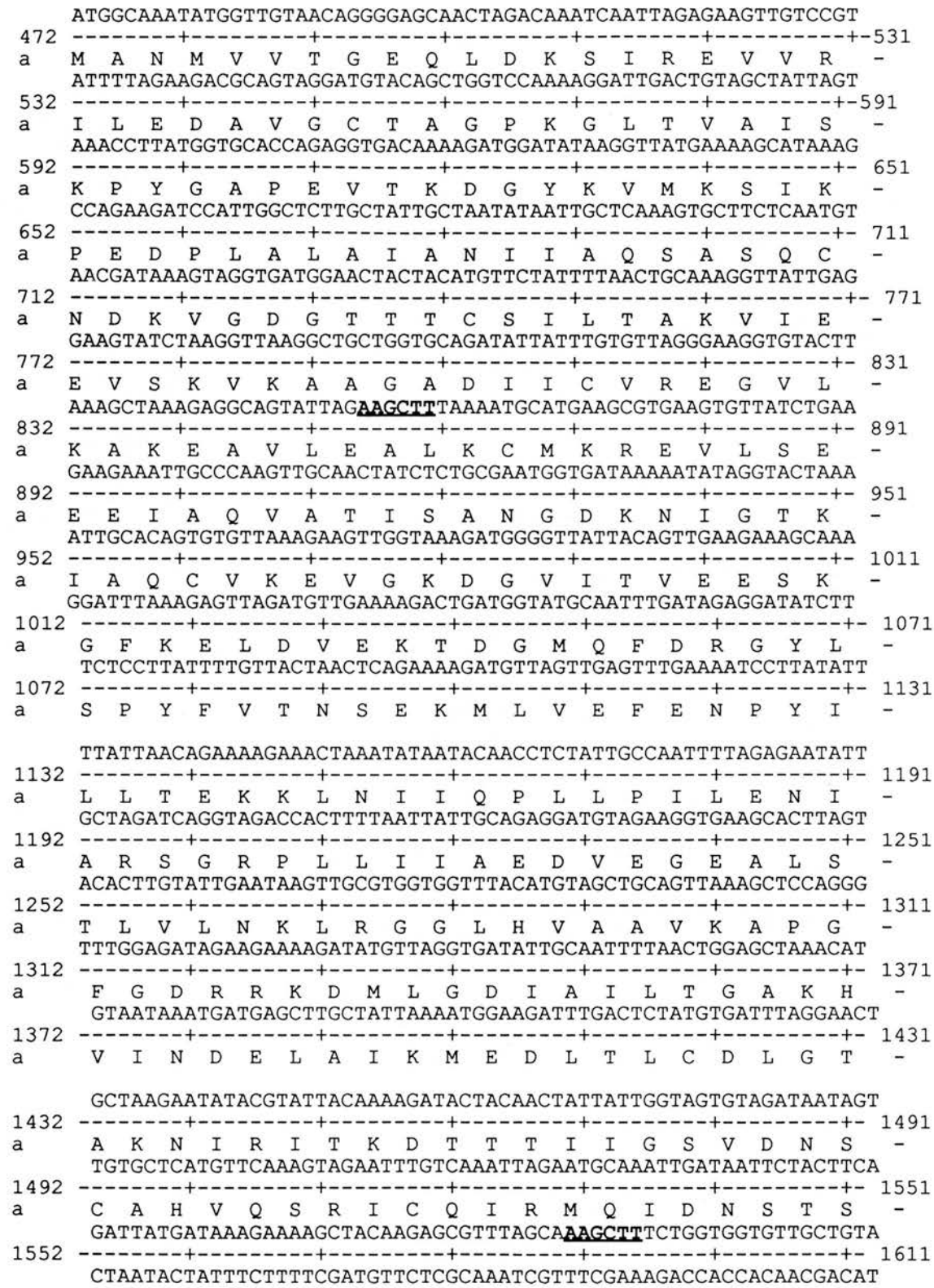



Figure 3.1 Amino (a) acid and nucleotide sequence of the *C.ruminantium* GroEL gene.The HindIII sites which were used for cloning the 0.7kb fragment into pTrHisHIII0.7 are indicated by the line below the HindIII recognition sequence (AAGCTT)in bold.


ATGGCAAATATGGTTGTAACAGGGGAGCAACTAGACAAATCAATTAGAGAAGTTGTCCGT
472 +-531
a M A N M V V T G E Q L D K S I R E V V R -
ATTTTAGAAGACGCAGTAGGATGTACAGCTGGTCCAAAAGGATTGACTGTAGCTATTAGT
532 +-591
a I L E D A V G C T A G P K G L T V A I S -
AAACCTTATGGTGCACCAGAGGTGACAAAAGATGGATATAAGGTTATGAAAAGCATAAAG
592 +- 651
a K P Y G A P E V T K D G Y K V M K S I K -
CCAGAAGATCCATTGGCTCTTGCTATTGCTAATATAATTGCTCAAAGTGCTTCTCAATGT
652 +- 711
a P E D P L A L A I A N I I A Q S A S Q C -
AACGATAAAGTAGGTGATGGAACTACTACATGTTCTATTTTAACTGCAAAGGTTATTGAG
712 +- 771
a N D K V G D G T T T C S I L T A K V I E -
GAAGTATCTAAGGTTAAGGCTGCTGGTGCAGATATTATTTGTGTTAGGGAAGGTGTACTT
772 +- 831
a E V S K V K A A G A D I I C V R E G V L -
AAAGCTAAAGAGGCAGTATTAGAAGCTTTAAAATGCATGAAGCGTGAAGTGTTATCTGAA
832 +- 891
a K A K E A V L E A L K C M K R E V L S E -
GAAGAAATTGCCCAAGTTGCAACTATCTCTGCGAATGGTGATAAAAATATAGGTACTAAA
892 +- 951
a E E I A Q V A T I S A N G D K N I G T K -
ATTGCACAGTGTGTTAAAGAAGTTGGTAAAGATGGGGTTATTACAGTTGAAGAAAGCAAA
952 +- 1011
a I A Q C V K E V G K D G V I T V E E S K -
GGATTTAAAGAGTTAGATGTTGAAAAGACTGATGGTATGCAATTTGATAGAGGATATCTT
1012 +- 1071
a G F K E L D V E K T D G M Q F D R G Y L -
TCTCCTTATTTTGTTACTAACTCAGAAAAGATGTTAGTTGAGTTTGAAAATCCTTATATT
1072 +- 1131
a S P Y F V T N S E K M L V E F E N P Y I -
TTATTAACAGAAAAGAAACTAAATATAATACAACCTCTATTGCCAATTTTAGAGAATATT
1132 +- 1191
a L L T E K K L N I I Q P L L P I L E N I -
GCTAGATCAGGTAGACCACTTTTAATTATTGCAGAGGATGTAGAAGGTGAAGCACTTAGT
1192 +- 1251
a A R S G R P L L I I A E D V E G E A L S -
ACACTTGTATTGAATAAGTTGCGTGGTGGTTTACATGTAGCTGCAGTTAAAGCTCCAGGG
1252 +- 1311
a T L V L N K L R G G L H V A A V K A P G -
TTTGGAGATAGAAGAAAAGATATGTTAGGTGATATTGCAATTTTAACTGGAGCTAAACAT
1312 +- 1371
a F G D R R K D M L G D I A I L T G A K H -
GTAATAAATGATGAGCTTGCTATTAAAATGGAAGATTTGACTCTATGTGATTTAGGAACT
1372 +- 1431
a V I N D E L A I K M E D L T L C D L G T -
GCTAAGAATATACGTATTACAAAAGATACTACAACTATTATTGGTAGTGTAGATAATAGT
1432 +- 1491
a A K N I R I T K D T T T I I G S V D N S -
TGTGCTCATGTTCAAAGTAGAATTTGTCAAATTAGAATGCAAATTGATAATTCTACTTCA
1492 +- 1551
a C A H V Q S R I C Q I R M Q I D N S T S -
GATTATGATAAAGAAAAGCTACAAGAGCGTTTAGCAAAGCTTTCTGGTGGTGTTGCTGTA
1552 +- 1611
CTAATACTATTTCTTTTCGATGTTCTCGCAAATCGTTTCGAAAGACCACCACAACGACAT

# CHAPTER FOUR

# CHARACTERISATION OF ELEMENTARY BODY ANTIGENS OF *C. RUMINANTIUM.*

## *4.1. Introduction.*

Animals which recover from natural infection with *C. ruminantium* or those immunised by infection treatment, attenuated organisms or with inactivated immunogens are immune to homologous challenge (Uilenberg; 1983; Jongejan, 1991; Martinez e*t al.,* 1993, 1994; Mahan *et al.,* 1995; Tafesse, 1992). The development of improved vaccines against heartwater has high priority because virulent blood stabilates and ground-up tick supernatants are cumbersome to use and expensive to produce (Oberem and Bezuidenhout, 1987a).

Characterisation of the antigenic components of *C. ruminantium* would make it possible to assess antigens that are important in immune response to this organism. The life cycle of *C. ruminantium* has two stages, an intracellular developmental reticulate body (RB) and an extracellular infective elementary body (EB) (Jongejan *et al.,* 1991c). It is thought that since the EB is the infective stage, its antigenic components, and in particular those which are on its surface may have functions associated with infection, may be targets for protective immune responses and could be used for developing subunit vaccines. Previous workers have identified antigenic components using antibodies raised in animals that have recovered from natural or experimental infections and animals that have been immunised with inactivated antigens. Their results describe antigenic components in general and not those on the

surface of the organism (Jongejan and Thielemans, 1989a; Rossouw *et al.,* 1990; Mahan *et al.,* 1993 van Kleef, Neitz and De Waal, 1992, 1993).

Detailed characterisation of antigenic components of pathogenic organisms requires them to be broken down into individual constituents which can be separated and analysed. A number of well established physical and chemical methods can be used, the choice of method depending on how the antigens are to be used. Physical methods include freeze thawing, freeze fracture and sonication. Chemical methods include those which utilise non-ionic and ionic detergents such as Triton X-100, Nonidet, and sodium dodecyl sulfate (SDS) and low osmolarity buffers to lyse the organisms. Physical and chemical breakdown of cells leads to the release of proteases, amino-peptidases and metalloproteases. Enzymatic activity can be controlled by addition of enzyme inhibitors (enzyme poisons) specific for classes of proteases. These include Aprotinin, (Trasylol) which acts against serine proteases, TLCK (tosyllysine-phenyl methyl sulfonyl fluoride) for serine and cysteine proteases and EDTA to inhibit metalloproteases. Solubilisation in solutions containing reducing agents such as dithiothreitol or mercaptoethanol, ionic detergents such as 1% SDS solution and boiling destroy protease activity. After extraction the released proteins have to be purified. The method used for purification depends on the purposes for which the antigens are to be used. Where the native state of the antigen should be retained, methods which do not require denaturing are employed. In situations where the native state of the antigen is not critical a method of purification which involves denaturing can be used. One of the most widely used methods for

separating proteins is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE method can be used to identify and monitor proteins during purification and also for assessing the homogeneity of the purified fractions (Garfin, 1990). The most commonly used SDS-PAGE is the discontinuous buffer system of Laemmli (1970), consisting of a separating and a stacking gel. Following SDS-PAGE the bands of fractionated antigens can be located, cut out of the gel, electroluted from the acrylamide and used. Denaturing and non-denaturing conditions were used in this study in order to compare their effects on the physico-chemical nature of *C. ruminantium* antigens.

The aim of experiments described in this chapter were to characterise the surface exposed antigenic components of *C. ruminantium* and to determine the effect of reducing and non-reducing conditions and heat on the mobilities of these antigens and their reactivity with antibodies from immunised animals.

## 4.2 Materials and Methods

### *4.2.1. Cowdria ruminantium stock.*

The Welgevonden stock (Du Plessis, 1985a) was used in this chapter. It was propagated in bovine endothelial cells as described earlier (Pow *et al.*, 1993, section 3.4).

### *4.2.2. Antisera.*

Sera obtained from two goats 28 days after immunisation by the infection and treatment method and sera obtained from two another goat obtained 34 days after

booster with inactivated elementary bodies as described earlier (Section 3.14), were used for detection of surface antigens.

### *4.2.3. Biotin labelling and detection of surface exposed proteins of C. ruminantium.*

Fresh EBs were obtained by harvesting 50 ml of culture supernatant of a 10 to 14 day culture. The EB pellet was suspended in either 0.05M sodium carbonate buffer (ELISA buffer) pH 9.6, or 0.1M sodium phosphate buffer ($NaH_2PO_4$) pH 7.0 in volumes sufficient to bring the protein concentration to 1mg/ml. The suspension of EB was further diluted to 50, 25, 12.5 and 6.25 μg/ml EB protein by mixing the suspension thoroughly, withdrawing 50 μl and adding it into 50 μl of fresh buffer. Unlabelled EBs were used as controls.

Biotinylation was achieved using two methods: first, EB dilutions prepared in sodium carbonate buffer pH 9 were mixed with 10 μg biotin (biotinamide caproate, Sigma, USA) dissolved in DMSO and incubated at room temperature for 4 hours. Second EB dilutions prepared in sodium phosphate buffer pH 7. 0 were mixed with 20 μg of Sulfo-NHS biotin (ImmunoPure® , Pierce, USA) and incubated for 30 minutes at room temperature before storage at -20°C until used. For SDS-PAGE analysis the method described in Section 3.8 was used with a few modifications. Briefly, the biotinylated EBs were dissolved in 2 times of sample buffer (0.68M Tris hydrochloride buffer pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5%, 2-mercaptoethanol and 0.05% bromophenol blue) and 10 μl per well of different concentration were loaded (2.5 μg, 1.25 μg and 0.625 μg/EB antigen per well).

The antigens were transferred onto nitro-cellulose membranes as described in Section 3.9. After two 5 minute washes in phosphate buffered saline containing 0.1% Tween 20 (PBST) the membranes were incubated with a 1/500 dilution of ExtraAvidin Peroxidase conjugate for 60 minutes with gentle agitation on a rocking platform. After three washes of 1 x 15 minutes and 2 x 5 mins, the blots were transferred into a container with a substrate solution (30 μg 4-chloro-1-N-naphthol in 10 ml ice cold methanol plus 50 ml, 20mM Tris buffered saline pH 7.5 with 30 μl ice cold hydrogen peroxide). Membranes were incubated for 5 to 10 minutes and the reaction was stopped by washing with distilled water after suitable colour development. The blots were examined for protein bands and the molecular masses of each band determined using a standard curve prepared by measuring the migration of the pre-stained standards. The blots were photographed using a Polaroid film (Type 55; Sigma, UK) in a Polaroid MP Land Camera with a 3A Kodak wratten filter.

### *4.2.4. Detection of the antigenicity of biotin labelled surface antigens of C. ruminantium using immune sera from I/T or IEB immunised goats.*

To identify that the biotin labelled proteins were also antigens recognised by antibody, SDS-PAGE and Western blotting of biotin-labelled EB samples was carried out as described in Section 4.2.3. The blots were then incubated in a 1/50 dilution of a day 28 PI serum from an I/T goat (G69) or with serum obtained on 34 PI serum from an IEB immunised goat (614) (Section 4.2.2). Then they were washed three times (2 x 5 minutes, 1 x 15 minutes) in PBST and incubated with a 1/500

dilution of peroxidase conjugate rabbit anti-goat IgG whole molecule (Sigma) in PBST for 60 minutes before colour development.

### *4.2.5. Determination of the effect of reducing agents and heat on unbiotinylated EB antigens of C. ruminantium.*

Frozen EBs were divided into two fractions for different treatment prior to SDS PAGE. They were mixed with equal volumes 2x sample buffer (Section 4.2.3) with or without the reducing agent β-mercaptoethanol (BDH, UK). Each sample was further subdivided into two fractions and one was heated for 5 minutes at 95$^{o}$C, while the other was kept at room temperature (22$^{o}$C). All the samples were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes as described earlier (Section 3.8 and 3.9). Detection of antigens was achieved by probing the blots with a 1/50 dilution of day 28 PI serum from I/T goat (G69) as described in Section 4.2.4. Other procedures and reagents are as described in Sections 4.2.3 and 4.2.4.

### *4.2.6. Fractionation of C. ruminantium antigens by SDS-PAGE.*

A large SDS-PAGE gel (16 cm x 18 cm x 1.5 mm) composed of 12% separating and 4% stacking gel was prepared as described in section 3.8 using a XL Protean II Dual slab apparatus (BioRad). A total of 32.5 μg of EB protein solubilised in 2x sample buffer and loaded into a single preparative well. Electrophoresis was performed at 200v/35mA for 1 hour until the sample was in the separating gel, and then at 65v/35mA overnight using a power pack model 1000/500(BioRad). The following day, the voltage was increased to 200v to

complete the electrophoresis, the gel was removed and kept in sterile de-ionised distilled ($ddH_2O$) water.

#### *4.2.6.1. Location of protein bands in SDS-PAGE gels.*

Protein bands were located by slicing a longitudinal strip (3 cm x 16 cm) from each side of the gel, one strip included the pre-stained protein markers. The strips were electroblotted onto nitro-cellulose membranes as described by Towbin, Staehelin and Gordon (1979) and the strip without the markers was immuno-blotted with goat antiserum. The blotted strips were placed alongside the gel to locate the position of protein bands.

Alternatively the whole gel was stained with copper stain using a copper staining kit (BioRad) according to the manufacturer's instructions. Briefly, gels were washed in distilled water, stained for 5 minutes, rinsed with de-ionised distilled water and proteins were visualised as dark bands by placing against a black background. Bands of interest were located and cut out of the gel and placed in universals. Six protein bands with molecular masses of 24kDa, 27kDa, 32kDa, 39kDa, 45kDa, and 66kDa were selected and sliced from the gel using a sterile scalpel blade. Destaining of the bands was carried out in two changes of a 1:10 dilution of copper destain for 5 minutes each and one change of a 1:20 dilution of destain for a further 5 minutes.

#### *4.2.6.2. Electro-elution of protein from polyacrylamide gels.*

Elution of proteins from the slices of polyacrylamide gels was carried out using a model 422 protein eluter (BioRad) following the manufacturer's instructions. Briefly each gel slice was placed in a glass tube fitted with a frit, silicone adapter and

membrane. The six tubes were placed in the electro-eluter assembly and the chamber was filled with electro-elution buffer (25mM Tris, 192mM glycine, 0.1% SDS) to a level just above the silicone adaptor. A magnet stir bar was placed into the chamber and the whole assembly was placed on a magnetic stirrer. Elution was carried out at 60mA (10mA per tube) for 5 hours with constant stirring.

The electro-eluted proteins were dialysed for 24 hours against 25mM Tris, 192mM glycine, 0.01% SDS. The eluates (600 µl) were removed from the dialysis membrane and placed in sterile Eppendorf tubes. SDS was removed from the eluates by overnight dialysis against distilled water at 4°C followed by desalting in a Nap 10 colunm® (Sephadex G25, Pharmacia, Sweden) according to the manufacturer's instructions as described in Section 3.8. Then the desalted eluates were freeze dried overnight using a Virtis freeze drier (Virtis, USA) and reconstituted in 100-µl of $ddH_2O$. Ten microlitre aliqouts were removed and analysed for purity by SDS-PAGE followed by Coomassie staining as described in Section 3.7. Samples containing protein were immuno-blotted to check for antigenicity. The remaining samples were kept at -20°C until they were used for lymphocyte proliferation tests (Section 6.2.3).

## 4.3. Results

### *4.3.1. Biotin labelling of surface antigenic components of the Elementary Body (EB) of C. ruminantium*

Six major protein bands were identified by both methods of biotin labelling (Figures 4.1a, 4.1b). These proteins had estimated molecular masses of 21kDa, 28kDa, 31kDa, 62kDa, 74kDa, and 115kDa. Several minor bands (15) appeared on the blots but they were clearer with Sulfo-NHS biotin due to reduced background staining (Figure 4.1b). Blots of the biotin-labelled proteins probed with immune sera from I/T or from IEB immunised goats revealed six major immunogenic proteins of the same molecular mass as those identified by biotin-labelling (Figure 4.2). Other bands with molecular masses between 14.5kDa to 142kDa were also recognised by immune serum.

### *4.3.2. The effect of reducing agents and heat treatment on the elementary body antigens of C. ruminantium.*

#### *4.3.2.1. Unheated and non-reduced samples.*

Five antigenic bands were present on blots of non-reduced un-heated EB samples (Figure 4.3, lane 2). Two bands migrating at 28kDa and 58kDa were prominent. The strongest reaction occurred with the 58kDa antigen. Other bands migrating at 31kDa and 14kDa were identified also. Two bands with higher molecular masses appeared faintly. Bands of relative molecular masses of 24kDa, 27kDa, 45kDa, did not appear in these blots.

#### *4.3.2.2. Heated and non-reduced samples.*

Eight dominant antigenic bands and three fainter ones were on blots of non-reduced and heated antigens (Figure 4.3, lane: 3). A band of 31kDa antigen was dominant, followed by 80kDa, 66kDa, 58kDa and 17kDa in order of descending intensity. Heating of non reduced samples led to appearances of more antigens migrating at higher molecular masses in particular a band migrating at about 80kDa was quite prominent. Fewer band migrating at lower relative molecular masses appeared on this blots. One band migrating between 20kDa and 28kDa was present.

#### *4.3.2.3. Heated and reduced samples of EBs.*

Western blots of heated and reduced EBs showed 14 antigenic bands with molecular masses ranging from 14kDa to 115kDa (Figure 4.3: lane 5). The major antigens were those with molecular masses of 24kDa, 27kDa, 31kDa, 45kDa, 51kDa, 58kDa, 66kDa and 80kDa. The other antigenic bands were faint. Heating and reducing generated more bands with lower relative molecular masses.

#### *4.3.2.4. Unheated reduced samples.*

Western blots with unheated but reduced EBs contained a total of 11 antigenic bands (Figure 4.4, lane 6). A band with a relative molecular mass of 28kDa was prominent in these blots. The other antigens of 18kDa 51kDa and 31kDa appeared but were fainter. The 31kDa antigen was less thick than in blots of reduced and heated antigens. Other bands appeared fainter than those seen on blots with the heated and reduced sample.

### *4.3.3. Electroelution of selected protein bands from SDS-PAGE gels.*

Western blot analysis of the eluted protein bands revealed two antigenic bands of 24kDa and the other of 31kDa (Figure 4.4 lane 1 and 3). Other eluted bands were contaminated with a 31kDa antigen. The 24 and 31kDa antigens were further tested in lymphocyte proliferation tests (Section 6.2.3).

## 4.4. Discussion

Surface exposed antigens of *C. ruminantium* EBs were identified by biotin labelling and Western blotting. Antigens which reacted strongly with antibodies in the serum of animals which had received either live or inactivated EBs had molecular masses of 24, 31, 45, 58, 66 and 80kDa. Three antigens of 27kDa, 66kDa and 115kDa reacted weakly. There was background staining on blots with biotin labelled proteins suspended in carbonate/bicarbonate buffer pH 9.6 which indicated that some protease activity had taken place. Protease activity was reduced considerably by using 0.1M sodium phosphate buffer pH 7.0 and Sulfo-NHS biotin. Antigens of the same molecular masses as those identified by biotin labelling have been termed as major antigenic peptides (Mahan *et al.,* 1994b) due to their strong reactions with antibodies from immune animals. Their strong reactivity with antibodies may indicate that they are involved in protective immune responses to *C. ruminantium.* This work has demonstrated for the first time that a number of the major antigenic proteins of *C. ruminantium* are located on the surface of the EB and that they are recognised by antibodies in serum from animals which have been immunised by I/T or with inactivated EBs of *C. ruminantium.*

The effect of a reducing agent in the sample was to increase the number of antigenic bands indicating that there may be 2-5 major antigens (polypeptides) on the surface of the EB. These antigens may be composed of proteins with extensive cross-linking, with perhaps 5 protein complexes or antigens present in 5 protein complexes which are composed of up to 14 peptide subunits of which the 28 and

58kDa are dominant. This situation has parallels with the structure of the chlamydial EB. In *Chlamydia* the outer membrane of the EB consists largely of disulphide cross-linked MOMP and two cysteine rich proteins (CRP) Storz and Kaltenboeck, (1993). The MOMP is not cross-linked on the reticulate bodies, and MOMP is identified as a pore-forming molecule whose activity is controlled by oxidation/reduction of disulphide bonds. Since *Cowdria* has a *Chlamydia*-like life cycle, with a similar pleo-morphic intracellular reticulate body stage, the effects of reducing agents suggests that cross-linked proteins constitute important components of the *Cowdria* EB. Cross linking of chlamydial proteins by disulphide bonds determines the rigidity of the EB envelope (Storz and Kaltenboeck, 1993). After entry of chlamydial EBs into the phagosome, the disulphide bonds are reduced, making the membrane more fluid and allowing development of the larger forms involved with multiplication (Storz and Kaltenboek, 1993).

The effect of heating was less marked, the migration of antigens was altered but the total number of bands/antigens in reducing or non reducing conditions was similar. These results indicate that tertiary and quaternary structures are present in *C. ruminantium* EB antigens. It is possible that the 14.5/15kDa antigen present in each treatment with similar migration may be similar to the LPS like antigen of 17kDa present in the rickettsiae (Ingalls *et al.*, 1995). LPS has been described in *Ehrlichia.* Lee and Rikihisa (1996) identified a periodate sensitive component on *Ehrlichia chaffeensis* important in monocyte responses. Periodate or protease treatments may be used to identify whether the 15kDa antigen here is the homologue of rickettsial

LPS. The antigenicity of the unreduced polypeptides indicated that they may have conformational epitopes in addition to the linear ones which were observed in heated and reduced samples.

Barbet *et al.* (1994) reported that the molecular mass of the 31kDa antigen was variable. In this study the 31/32kDa antigen was not dominant in blots with reduced but unheated samples, instead a 28kDa antigen was dominant, the presence of disulphide bonds between peptides, folding and non-covalent interactions could explain this observation.

*C. ruminantium* contains a complex mixture of extracellular and intracellular detergent soluble and insoluble antigenic components (Paxton, Sumption, Lally, and Pow, 1993, unpublished data). Among the 6 surface exposed antigens identified in this study, the 28kDa is soluble, while the 21 and 31kDa are present in the soluble and insoluble fractions. These antigens are also cross reactive with sera from animals infected with *Ehrlichia phagocytophila* and *E. ondiri.*

Rossouw *et al.* (1990) identified 20 antigenic components of the Welgevonden stock, 12 in the Ball 3 reference stock and 9 for the Kwanyanga stock using homologous and heterologous antisera from immunised animals. They reported that two antigens of 27kDa and 31kDa were common in all of antigenic profiles of the 9 stocks they examined. In this study two antigens of 28kDa and 31kDa were identified on the surface of the EB and it is likely that they are the same as those reported by the above workers.

*C. ruminantium* antigens of 21kDa, 32kDa, 40kDa, 45kDa, 58kDa, 85kDa and 160kDa have been referred to as major antigenic peptides (Mahan *et al.*, 1994b) and are shared between stocks. Among these antigens the 21, 32 and 58 are on the surface of the EB. The 32kDa Major Antigenic Protein 1 (MAP1) formerly referred to as Cr32 (Jongejan and Thielemans, 1989a; Rossouw *et al.*, 1990) is immunodominant and conserved among several stocks of *C. ruminantium* and *Ehrlichia* species (Jongejan *et al.,* 1989). The methods used to prepare these antigens did not affect their reactivity to antibody. Antigens were prepared from crude tissue extracts of infected animals (Jongejan and Thielemans, 1989a), infected cell suspensions (Rossouw *et al.*, 1990) and from pure elementary bodies (Paxton *et al.*, 1993). In all cases it was possible to separate them by SDS-PAGE and identify them with polyclonal antisera obtained from infection-treatment animals (Paxton *et al.*, 1993), or those immunised with inactivated immunogens emulsified in an appropriate adjuvant (Jongejan and Thielemans, 1989a; Rossouw *et al.*, 1990). In this study surface exposed antigenic components of the elementary body of *C. ruminantium* were identified by biotin labelling and it was demonstrated that specific antibodies to them are developed by animals which have received live or inactivated immunogens respectively. The role played by these antigens in protective immune responses to *C. ruminantium* is not known. In view of their location and strong reactions with antibody these proteins may be targets for inducing protective immunity individually or in various combinations. Immunisation with the reduced 32kDa antigen which is a major structural protein (Mahan, 1995) does not confer any

protection on animals (van Vliet *et al.*, 1994). Immunisation with the surface 21kDa antigen has not been attempted.

The 58kDa antigen probably belongs to the family of heatshock proteins (Hsp) designated Hsp 10 and Hsp 60. Hsps are highly conserved among eukaryotic and prokaryotic organisms and are major targets of antibody responses (Kaufmann, 1993). The gene coding for the 58kDa protein termed GroEL has been cloned and expressed in *E. coli* (Lally *et al.*, 1995) and immune responses to the recombinant 58kDa Hsp in sheep and mice were investigated (Chapter 7). Two other recombinant antigens have been produced the MAP1 (van Vliet *et al.*, 1994) and 21kDa proteins (Mahan *et al.*, 1994b). The value of these antigens for diagnostic purposes and immunogenicity has being investigated in other laboratories (van Vliet *et al.,* 1994; Mahan *et al.*, 1994b).

Individual antigenic proteins have been used successfully to induce protective immunity in other rickettsial diseases. Two protein antigens of A*naplasma marginale* with molecular masses 105kDa (Palmer, *et al.*, 1986) and 36kDa (Palmer, *et al.*, 1988) have been used to induce protective immunity to homologous and heterologous challenge. Identification of surface protein antigens of *C. ruminantium* could lead to the isolation of cross-protective antigens for immunisation and for the development of specific serological tests. This is particularly important in situations where cross-reactions occur between closely related organisms due to the presence of shared epitopes among conserved antigens (Jongejan, *et al.*, 1993a). This study has demonstrated that several of the major immunogenic antigens of *C. ruminantium* are

located on the surface of the EB and the fact that it was possible to purify some of these antigens by SDS-PAGE enables the investigation of their role in immune responses to heartwater.

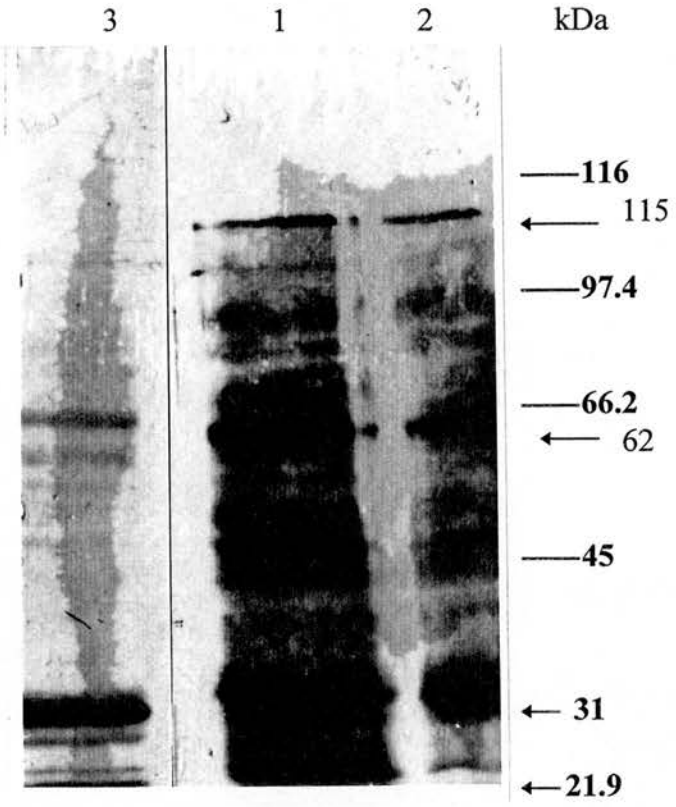
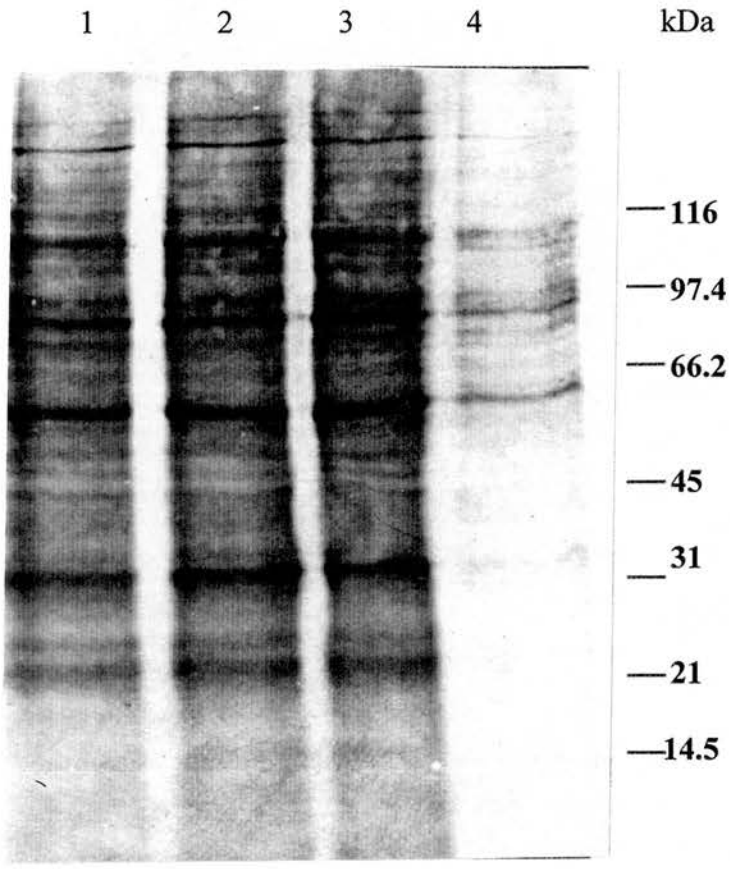



Figure 4.1a. Biotin labelled surface EB proteins of *C. ruminantium*.
Lanes were loaded with proteins in carbonate buffer (pH 9.6) as follows: lane 1, 50 μg/ml of EB protein; lane 3, 12.5 μg/ml and lane 2, 25 μg/ml of EB protein. The arrows indicate bands of dominant surface proteins.




Fiigure 4.1b. SDS-PAGE of biotin labelled surface proteins of the EB of *C. ruminantium.*
Lanes were loaded with antigens in sodium phosphate buffer (pH 7.0) as follows: lanes 1 to 3, 50 μg/ml of of EB protein and lane 4 with 25 μg/ml EB antigen.

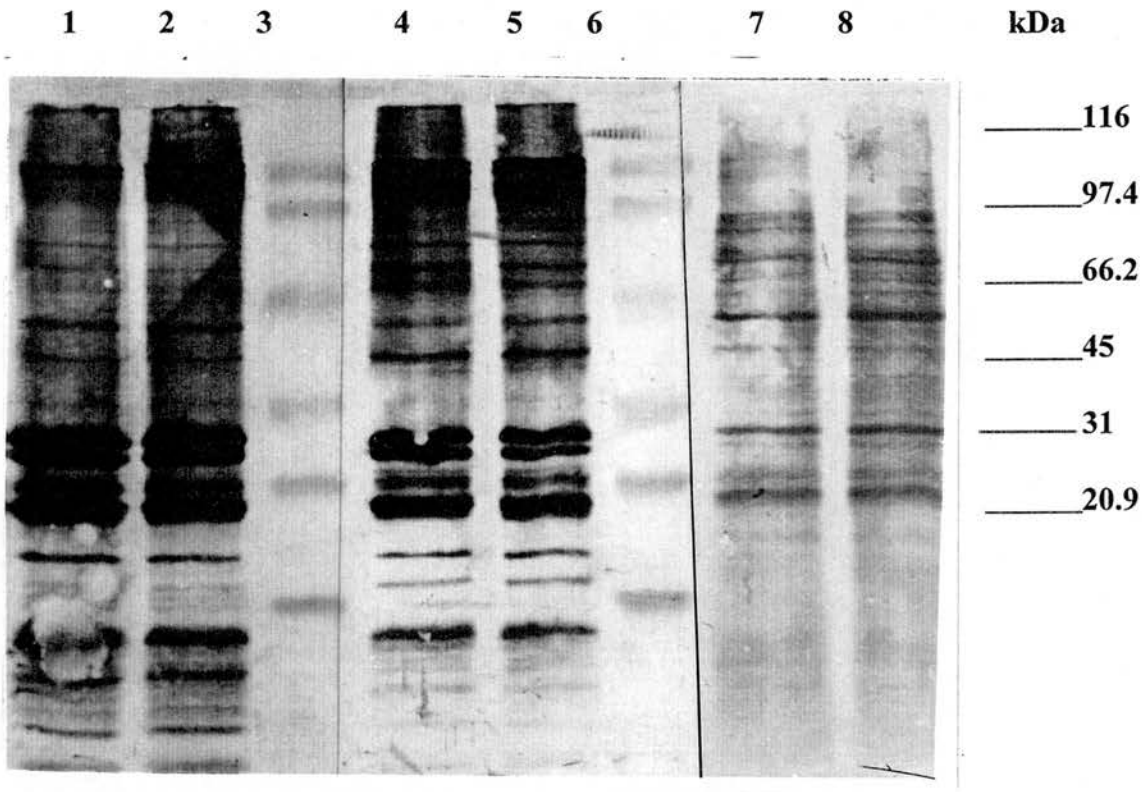



Figure.4 2. Western blots demonstrating recognition of biotin labelled EB proteins of *C. ruminantium* by antibodies in serum of goats immunised with live (I/T) and inactivated EBs (IEB).
Lanes 1 and 2, immunoblots of EB proteins tested with day 28 PC serum from an I/T goat G69; lane 3 and 6, SDS protein standards; lanes 4 and 5, were tested with day 34 serum from IEB goat (G614); lanes 7 and 8, tested with ExtraAvidin peroxidase.

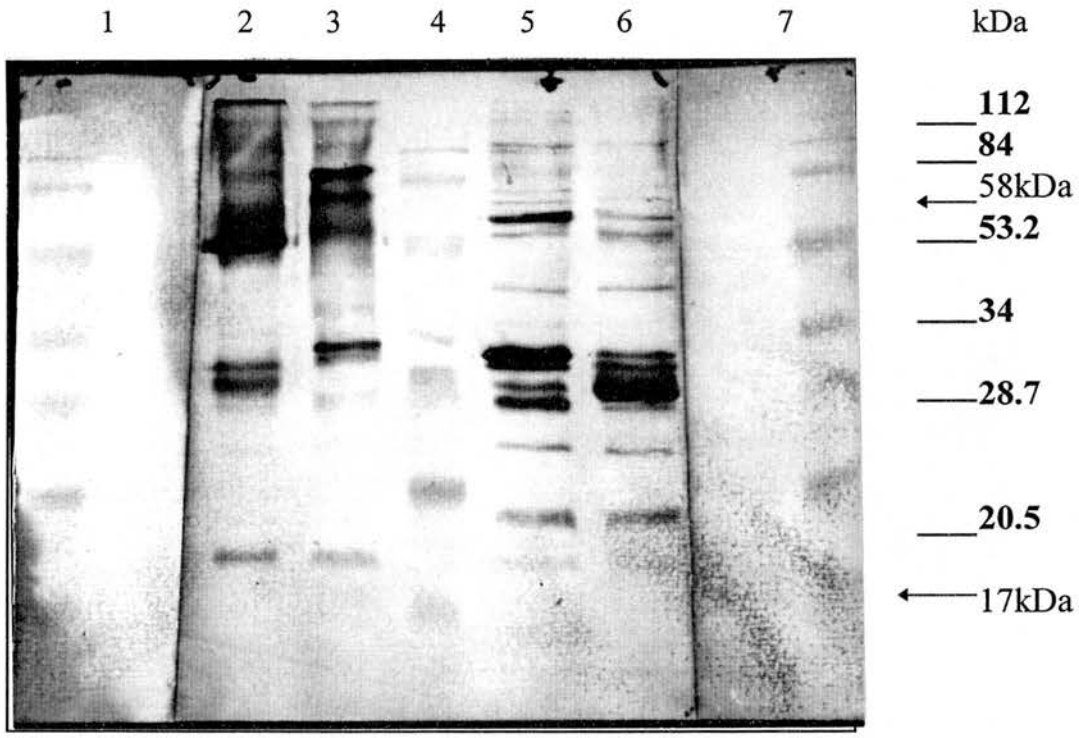



Figure 4.3. Western blot analysis of *C. ruminantium* EBs showing the effect of reducing and non reducing conditions with and without heating.
Lane 1, SDS-PAGE protein standards; lane 2, non-reduced and un-heated EB antigens; lane 3, heated non-reduced antigens; lane 4, protein standards, lane 5, reduced heated antigens; lane 6, reduced un-heated antigens; lane 7, protein standards.

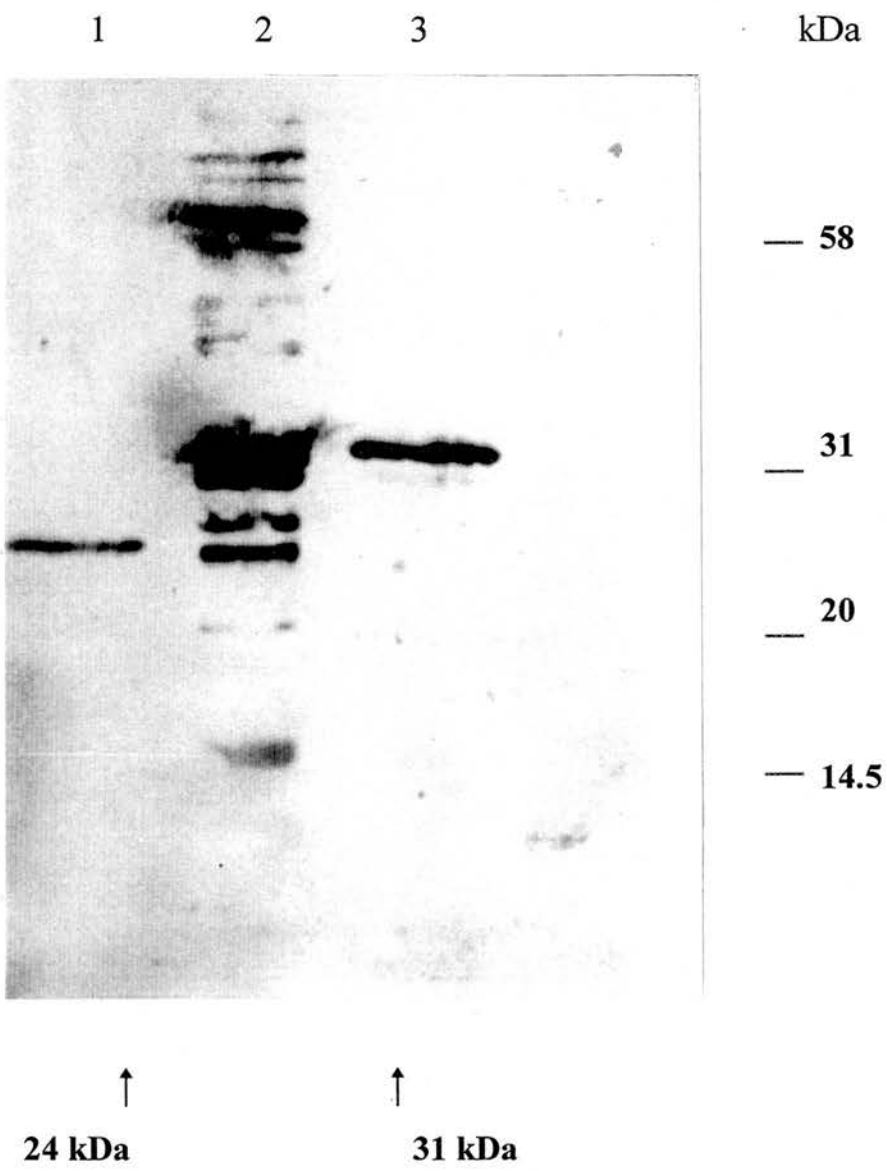



Figure 4.4. Western blot analysis of SDS-PAGE purified antigens of *C. ruminantium* blots were tested with day 28 sera from I/T goat (G69). Lane 1, 24kDa SDS purified antigen; lane 2, control containing EB antigens of *C.ruminantium* and lane 3, SDS-purified 32kDa antigen.

# CHAPTER FIVE.

## IMMUNOGLOBULIN G RESPONSES OF RUMINANTS AND MICE TO IMMUNISATION WITH LIVE AND INACTIVATED ANTIGENS OF *C. RUMINANTIUM*

### *5.1. Introduction*

Animals which recover from heartwater infection develop antibodies to *Cowdria ruminantium* (Neitz *et al.*, 1986; Du Plessis and Malan, 1987b; Semu *et al.,* 1992).

Detection of the antibody responses of naturally infected ruminants and those inoculated with either live *C. ruminantium* or with inactivated EBs can be carried out by a number of serological tests. These include the indirect fluorescent antibody test (IFAT) (Du Plessis, 1981; Brown, *et al.,* 1989; Du Plessis *et al.*, 1993b; Lawrence *et al.*, 1993), the enzyme linked immunosorbent assay (ELISA) either as an indirect assay (Neitz *et al.*, 1986; Soldan *et al.*, 1993) or as a competitive assay ( Du Plessis *et al.*, 1993 Jongejan *et al.*, 1991a), and Western blotting (Jongejan and Thielemans, 1989a; Rossouw *et al.,* 1990; Mahan *et al.*, 1993).

Circulating specific antibodies to *C. ruminantium* were detectable by indirect ELISA in serum of goats 10 days after experimental infection or immunisation with inactivated elementary bodies of *C. ruminantium* (Tafesse, 1992; Viljoen *et al.*, 1987). In mice antibodies were detected 14 days post inoculation by IFAT (Byrom *et al.*, 1993). IFAT has been used for the diagnosis and monitoring of serological response to heartwater immunisation (Lawrence *et al.*, 1993, 1995) and ELISA has been used for epidemiological studies (Kobold *et al.*, 1992). However cross-

reactions with *Ehrlichia* spp. are a drawback to the use of crude antigen preparations (Du Plessis Boersema and van Strijp, 1994). Western blotting is used for the identification of specific antigenic components of *C. ruminantium* which may have a role in protective immunity to the host (Jongejan and Thielemans, 1989a; Rossouw *et al.*, 1990; Mahan *et al.*, 1993).

There are four major immunoglobulin classes in the ruminant, IgG, IgM, IgA and IgE. Immunoglobulin M appears early (9-12 days) after infection with heartwater with titres peaking between 15 to 18 days (Afonso, 1995). IgG appears about the same time as IgM but it peaks around 28 days post infection and persists for longer periods than the other immunoglobulin classes. There are two major subclasses of ruminant IgG, $IgG_1$ and $IgG_2$ of which $IgG_1$ is the predominant in sheep (approximately 2/3 of total serum IgG) (McQuire, Musoke and Kurth, 1979). Immunisation of sheep with protein antigens generates antibody responses of both isotypes (Bird *et al.*, 1995). The role played by the IgG isotypes in immunity to pathogenic organisms has been investigated by several workers. For example, $IgG_2$ is important in protection against *Staphylococcus aureus* mastitis in sheep (Watson, 1988). There are two high affinity FcγR receptors on ruminant cell surfaces, one for $IgG_2$ and the other for $IgG_1$. High affinity receptors for $IgG_2$ are found on neutrophils and monocytes/macrophages, while those with high affinity for $IgG_1$ are expressed on alveolar macrophages and cultured monocytes (Howard, Taylor and Brownlee, 1980). The major effector cells of antibody-mediated cytotoxicity (ADCC) in the ruminant are neutrophils and monocyte/macrophages (Grenwal and Rouse, 1979).

Immunisation of mice with soluble protein antigens leads to two types of $CD4^+$ T helper lymphocyte responses referred to as Th1 and Th2 that are differentiated by the cytokines that are produced (Mosmann and Coffman, 1989).

In Th1 type responses $CD4^+$ T cells produce interferon gamma (IFN-$\gamma$), interleukin-2 and tumor necrosis factor alpha (TNF-$\alpha$). Their effect is to promote cell-mediated immunity and support production of $IgG_2$ antibodies. In Th2 type responses $CD4^+$ T cells secrete IL-4, IL-5, IL-6 and IL-10. Their effect is to support IgE, $IgG_1$ and IgA production and to promote eosinophilia. Immunity to intracellular bacteria is thought to be mediated principally by cell-mediated immunity. The IgG isotype antibody which is associated with these infections would be $IgG_2$. However this seems to be only true for some infections caused by some intracellular bacteria. Dominance of $IgG_1$ or $IgG_2$ responses in ruminants infected naturally or experimentally with certain intracellular bacteria or those vaccinated has been reported in cattle and goats. Schmeer, Schnorr, Perez-Martinez and Storz (1987) used indirect ELISA to differentiate *Chlamydia psittaci* specific $IgG_1$ and $IgG_2$ levels in serum samples from cattle with natural chlamydiosis and those which were experimentally infected. The mean $IgG_1$:$IgG_2$ ratios of the naturally infected cattle was 1:4 indicating a significant $IgG_2$ dominance, similar ratios were obtained with serum from experimentally infected cattle. In contrast significant $IgG_1$ but lower $IgG_2$ levels were associated with natural *Coxiella burnetti* infections, but vaccination of cattle with formalin inactivated *C. burnetti* vaccine led to the development of a highly significant $IgG_2$ response (Schmeer, Kraus, Lorbach and Weigand, 1986). Natural infections of

*Chlamydia psittaci* in goats were characterised by high $IgG_1$ responses whereas vaccination led to mixed responses of $IgG_1$ and $IgG_2$ although $IgG_1$ was predominant (Schmeeer, *et al.*, 1987). Vaccination of goats with inactivated *C. burnetti* produced a dominant $IgG_2$ response. Butler, Seawright, McGivern and Gilsdorf (1981) demonstrated that $IgG_1$ was dominant in sera from infected cattle, after immunisation and subsequent challenge with *Brucella abortus* S19.

IgG isotype predominance in virus infections of sheep has also been demonstrated. Bird *et al.* (1995) demonstrated dominance of $IgG_1$ in serum from sheep infected with the Maedi-Visna virus, they also found that sheep infected with the parapox orf virus developed a predominant $IgG_2$ response.

*Cowdria ruminantium* has a life cycle similar to that of the obligate intracellular rickettsia *C. psittaci*, and given this similarity I examined the IgG whole molecule, $IgG_1$ and $IgG_2$ responses of cattle, sheep, goats and mice to *C. ruminatium* EB antigens following experimental infection (I/T), field challenge, and immunisation with inactivated EBs or recombinant antigens.

## 5.2. Materials and Methods

### *5.2.1. Antigens.*

The antigens used for Western blotting and ELISA tests in this chapter were from Elementary bodies (EBs) of *C. ruminantium* (Welgevonden stock). Two recombinant antigens, (Ags) a 58kDa Hsp and its subclone a 35kDa protein were also used. The 35kDa recombinant protein was expressed in *E. coli* and purified as

described in Section 3.7 and used for ELISA tests to detect IgG1 and IgG2 responses of mice and sheep immunised with the 58kDa recombinant Ag.

***5.2.2. Sera.*** Sera from five groups of goats, two groups of sheep, 3 groups of cattle and three groups of mice were examined for *Cowdria* specific IgG antibodies by Western blotting and ELISA.

***Caprine sera***

Goats in groups C1, C2 and C3 (6 goats each) were part of a previous vaccine trial (Tafesse, 1992). They had been immunised with inactivated EBs (IEB) or detergent extracted soluble antigens followed by challenge with live virulent blood stabilate.

Goats in group C1 were inoculated once with IEBs and those in group 2 were inoculated 2 times with IEBs 21 days apart. Antigens used for primary immunisation were mixed with equal volumes of FCA and inoculated intramuscularly and those used for booster were mixed with equal volumes of FIA or PBS as described earlier in Section (3.14) and administered subcutaneously 21 days later. Goats in group C3 were treated as those in group C2 except that they had been inoculated with detergent extracted EB antigens.

Sera were collected sequentially from each group at days 0, 7, 14, 21, 28, and day 34 PI. On day 36 PI, 3 goats from each group were challenged by intravenous inoculation of 5 ml of virulent blood stabilate of the Welgevonden stock. Four of the goats survived challenge and serum was collected at day 28 post challenge (PC) (which is day 64 PI). Detection of *Cowdria* specific IgG antibodies was carried out

using 8 sera (5 from group 1 and 3 from group 2) collected after immunisation (= day 34 PI) and 4 sera collected at day 28 PC (= day 64 PI) from 4 survivors of the challenge and 3 sera obtained day 34 PI from group 3.

Sera in the fourth group (group C4) came from two goats which were as part of an I/T immunisation study (Section 4.2.2) Two serum samples collected at day 28 and day 80 post infection were examined for *Cowdria* specific $IgG_1$ and $IgG_2$ antibodies.

Sera in the fifth group (group C5) were from 2 goats (G107, G108) immunised with IEB as described in Section 3.14.1. Western blot analysis was used to examine sequential sera collected from these goats on day 0, 14, 21, and day 28 PI.

The control sera from an I/T goat (G69 day 28 PI)) and from an uninfected goat (G106) were included in all tests.

***Ovine sera***

Group S1. There were 6 sera in this group obtained from three sheep infected with the Mara and Nonile stock of *C. ruminantium*. They were obtained at day 0, 14 and 28 after infection.

Group S2. Sequential serum samples obtained post immunisation (day 0, 14, 21, 28, 64, and day 143 PI) from 6 sheep immunised with 58kDa Hsp recombinant antigen and from 6 controls (Section 3.2) were examined for *Cowdria* specific $IgG_1$ and $IgG_2$ responses to recombinant 35kDa subclone antigen the 58kDa Hsp of *C.ruminantium.*

***Bovine sera.***

Sera from three groups of cattle were examined for *Cowdria* specific $IgG_1$ and $IgG_2$ antibodies. Sera in group 1 and 2 were donated by Dr. J. Du Plessis of Onderstepoort Institute South Africa and sera in group 3 were donated by Dr. Philipe Totte from International Research Institute (ILRI) Nairobi Kenya.

Group B1. Sera were raised by experimental infection using blood stabilates. There were seven sera in this group, which were obtained from 7 cattle immunised by I/T with Ball 3, Kwanyanga and Mara stocks of *C. ruminantium*.

Group B2. Sera came from animals which received field exposure. There were 10 sera in this group. They were collected from cattle (B196, B199, B200 to B206, B210 and B211) in a heartwater endemic area of South Africa.

Group B3. Sera in this group were from cattle immunised with IEB's and subsequently challenged. There were 13 sera in this group which came from a vaccine trial conducted in Kenya (ILRI) by Dr. Philippe Totte. Nine sera were obtained from 3 cattle (S295, S296, and S304) immunised with IEBs of the Gardel stock. They were collected at day 0, 36 PI and at day 31 PC. The remaining 4 sera were from two control cattle (S234 and S264). They were collected at day 0 and day 11 PC.

***Murine sera***

Sera from 4 mice infected with *C.ruminantium* then treated (I/T) and from 5 mice immunised with IEBs were collected the same occassion (day 34 PI) post

immunisation and examined for $IgG_1$ and $IgG_2$ antibody responses to *C. ruminantium*.

Pooled sequential mouse sera (day 0, 14, 21 and day 34 PI) obtained from mice which had been immunised with recombinant 58kDa Hsp (Section 7.2.1.2, 7.2.1.4, Table 7.1) were also examined for $IgG_1$ and $IgG_2$ antibodies to a recombinant 35kDa subclone protein of *C. ruminantium* by ELISA.

### *5.2.3. Detection of Cowdria antigens recognised by antibodies in goat sera by Western blotting.*

Western blot analysis was used to determine antigens recognised by antibodies in sera obtained after immunisation from goats in groups C1, C2, C3, C4 and C5. It was carried out as described in Section 4.2.4. The Molecular masses of the bands were estimated as described in Section 4.2.3 and the blots were photographed for permanent record as described in Section 3.11.

### *5.2.4. Detection of $IgG_1$ and $IgG_2$ isotype responses of goats, sheep and mice immunised with C. ruminantium antigens by indirect ELISA.*

The method given below is a modification of that described by Soldan *et al.*, (1993) to enable specific isotype responses to be identified. 96-well flat-bottomed microtitre ELISA plates (Immulon 1, Dynatech Laboratories) were coated with 50µl of a 1/1000 dilution of ELISA antigen in coating buffer (0.05M carbonate/bicarbonate buffer pH 9.6). The plates were covered with cling film and incubated at 4$^{o}$C overnight. The contents were discarded and the plates were washed three times (3 mins/wash) in 0.9% sodium chloride containing 0.05% (v/v) Tween 20 (0.9% NaCl/PBST). After washing, the plates were incubated with 100 µl per well

of 4% normal rabbit serum in PBS containing 0.05% Tween 20 (PBST/4% NRS) for 1 hour at room temperature and the buffer was discarded. Fifty microlitres of sera diluted at 1/50 in blocking buffer were added to the wells, the plates covered with cling film and incubated at 37°C for 1 hour. After 3 washes, 50µl of rat anti-sheep $IgG_1$ (1RS) or $IgG_2$ (2RS) monoclonal antibody diluted to 1/20 in blocking buffer was added to all wells prior to incubation at 37°C for 1 hour. For tests with mice sera, rat anti-mouse $IgG_1$ and $IgG_2$ monoclonal antibodies (Serotech, UK) were used. The plates were washed 3 times as above. This was followed by the addition of 50 µl of goat anti-rat IgG whole molecule HRP (Sigma) conjugate diluted 1/1000 dilution in blocking buffer to all wells and incubated at 37°C 1 hour. The plates were washed 3 times and 50 µl of the peroxidase substrate, tetramethyl benzidine (TMB, Kirkegaard and Perry laboratories) was added to all wells. The reaction was stopped after 15 minutes by the addition of 50 µl of 0.2M sulphuric acid. The absorbance (Optical density/ODs) at 450 nm were read using a plate reader (Multiscan plus, Version 2.03, Labsystems). The mean of 2 tests was calculated, then the OD of the negative control was subtracted from this value to remove background absorbance. The results were expressed as the mean OD value of 2 tests

### *5.2.5. Detection of IgG isotype responses of immunised goats to C. ruminantium by Western blotting.*

EBs of the Welgevonden stock of *C.ruminantium* were used as antigens. SDS-PAGE and Western blotting was carried out as described in Sections 3.5 and 4.2.5 with the following modifications to enable specific isotype responses to be detected:

Following blocking of the membranes and incubation with primary sera, the blots were reacted with monoclonal antibodies to sheep $IgG_1$ and $IgG_2$. The monoclonal antibodies (IRS and 2RS) were in culture supernatants obtained from Dr. John Hopkins, Department of Veterinary Pathology, University of Edinburgh and were specific for isotypes $IgG_1$ and $IgG_2$ respectively.

Optimal working dilutions of each MoAb used in Western blot analysis were established to be 1/10 for 2RS and 1/20 for 1RS. Following incubation for 60 minutes colour was developed in the usual way.

### *5.2.6. Detection of IgG isotype responses of cattle to C. ruminantium by indirect ELISA.*

The method used was a modification of that used for goat sera (section 5.2.4).

Mouse anti-bovine $IgG_2$ monoclonal was obtained from Sigma (Sigma, UK), and HRP conjugated sheep anti-bovine $IgG_1$ antibody was obtained from Bethyl Laboratories (USA). The working dilutions of the antibodies were determined by carrying out a checkerboard titration.

For performance of the test proper volumes of 100 µl per well for all reagents were used, antigen dilutions of 1/4000 and 1/6000 in carbonate buffer pH 9.6 for Gardel and Welgevonden respectively was used to coat the plates. Plates were blocked for 1 hr at room temperature with PBST containing 4% Normal goat serum (PBST/4% NGS). Plates were washed manually 3 times in PBS, with 0.05% Tween (3 minutes each).

The plate was divided into two equal halves. Wells in columns 1-6 of rows A to G were used to screen sera for $IgG_1$ and wells in columns 7-12 used for screening for $IgG_2$. Wells in row H were used for the controls. Each test was incubated in duplicate using serum dilutions of 1/800 prepared in PBS containing 4% NGS serum. Mouse anti-bovine $IgG_2$ monoclonal antibody (Sigma) was used at 1/10,000 to detect $IgG_2$ responses, then goat anti-mouse IgG whole molecule HRP conjugate (Sigma) was used at 1/2000 to detect binding of the monoclonal antibody. Sheep anti-bovine $IgG_1$ HRP conjugated polyclonal serum (Bethyl Labs) was used at 1/50,000 to detect $IgG_1$. All dilutions were carried out in blocking buffer. The chromogen, reaction times and stopping of the tests was as described earlier (Section 5.2.4).

### *5.2.7. Statistical analysis.*

The student's t test was used to analyse for differences between IgG1 and IgG2 antibody responses in sera from IEB immunised goats pre-challenge and post challenge.

## *5.3 Results.*

### *5.3.1. Antigens of Cowdria elementary body recognised by immune sera*

***Antigens recognised by antibodies after I/T immunisation.***

Sera from goats immunised by live (group C4) reacted to between 5 and 7 antigens in Western blots of the EB (Figure 5.1, lanes 2 and 3). The 5 predominant antigens were of molecular masses 21kDa, 24kDa, 28kDa, 32kDa, and 58kDa. A 66kDa antigen was usally observed but was faint.

***Antigens recognised by antibodies following immunisation with IEBs.***

Goats immunised with inactivated EB's (groups C1, C2 and C5) had antibodies by the 14 day post immunisation which recognised at least 5 to 6 antigens of the EB (Figure 5.1, lanes 4, to 7 and Figure 5.2, and 5.3 lanes 1, to 3, and 5, Table 5.1). The predominant 5 antigens were 21kDa, 24kDa, 28kDa, 32kDa, and 58kDa.

Sequential sera from two IEB immunised goats (group C5) showed positive reactions with five EB antigens of *C. ruminantium* from day 14 PI onwards (Figure 5.2). These sera detected antigens with molecular masses of 24kDa, 27kDa, 28kDa 32kDa and 58kDa. Sera from G108 reacted strongly with the 58kDa antigen and weakly with the 66kDa antigen (Figure 5.2 lanes, 6 and 7). The reactions of serum from G107 were weaker than those from G108 (Figure 5.2 lanes, 2 and 3).

***Antigens recognised by antibodies following live infection (challenge) of IEB immunised goats.***

Post-challenge sera from 4 goats in groups C1 and C2 (G476, G601, G614 and G668) which survived challenge developed antibodies which reacted with 4 to 5 more antigens than those recognised by post immunisation sera from the same

animals (Figure 5.4 lanes:1, 2, 3, and 4, Table 5.1). Sera from one goat (G614) had antibodies which reacted with up to 15 antigens (Figure 5.4 lane: 2 indicated by the arrow). The antigens which were recognised by antibodies in these sera but not by pre-challenge sera from the same animals were of molecular masses 15kDa, 36kDa, 45kDa, 66kDa, and 80kDa. Weaker reactions were also observed with antigens of higher molecular masses.

Sera obtained from 2 goats (G614 and G668) at 18 mo and 24 mo post-challenge recognised between 7 and 15 antigens of the EB (Figure 5.5). Sera collected 18 mo and 24 mo post challenge from G614 reacted with at least 15 antigens with molecular masses similar to those of day 28 PC (Figure 5. lanes 5 to 8). Antibodies in sera from goat G688 reacted with at least 9 antigens (Figure 5.5 lanes: 8 to 12). The antigens recognised by these antibodies were between molecular masses 15kDa and 80kDa.

***Antigens recognised by antibodies following immunisation with soluble antigens (Sags).***

Three antigens were detected by sera from goats immunised with soluble antigens (group C3) (Figure 5.1, lanes 8 and 9, Figure 5.3 lanes 7 and 8, Table 5.1). These antibodies reacted strongly with a 32kDa antigen and weakly with antigens of 21kDa, 24kDa, and 28kDa.

The four antigens detected by sera from all 4 groups were those with molecular masses of 24kDa, 27kDa, 28kDa, and 31 kDa. Two additional antigens with

molecular masses of 58kDa and 66 kDa were detected by sera from I/T and IEB immunisation.

### *5.3.2. $IgG_1$ and $IgG_2$ responses goats of immunised by I/T or with IEB's.*

***$IgG_1$ and $IgG_2$ responses of immunised goats measured by ELISA.***

Goats immunised with inactivated EB's developed a predominant $IgG_1$ response before challenge (Figures 5.6 lanes and Table 5.3). There were significant differences between the ELISA OD values of $IgG_1$ and $IgG_2$ isotypes following IEB immunisation ($t=3.182$, $p<0.05$). $IgG_1$ was dominant in these responses. The $IgG_1$:$IgG_2$ ratios for 4 goats in groups 1 and 2 were 5:1 for (G476), 2:1 (G614, G668) and 1.5:1 (G601) (Table 5.2).

After live challenge of the IEB immunised goats, the level of $IgG_2$ isotype as measured by OD value in ELISA, rose to between 7 and 10 times their pre-challenge levels and $IgG_1$ ODs in post challenge sera increased by a factor of 3 to 6 times (Figure 5.6). Analysis for differences between $IgG_2$ responses pre-challenge and post-challenge indicate that the levels of $IgG_2$ had increased after challenge ($t=2.353$, $p<0.05$).

The rise in $IgG_2$ had the result that the differences in $IgG_1$ and $IgG_2$ levels was no longer significantly different ($t=3.182$, $p>0.05$). After challenge the ratio between $IgG_1$ and $IgG_2$ responses had changed to between 2:1 to 1:1.6.

In contrast to to the effect of live challenge on IEB immunised goats the responses to live infection (I/T) of naive goats (G69, G74 group 4) resulted in a very marked $IgG_1$

response and no significant detection of $IgG_2$ when sera collected on days 28 and 80 were tested.

The $IgG_1$ and $IgG_2$ responses of sheep (group S2) immunised with recombinant antigens were similar to those of sera from I/T goats and those of pre-challenge from IEB immunised goats. They were characterised by higher $IgG_1$ OD values and an $IgG_1$:$IgG_2$ ratio of 2:1.

***$IgG_1$ and $IgG_2$ antibodies in post immunisation and post-challenge sera from IEB immunised goats recognise specific antigens of the EB in Western blots.***

To confirm that $IgG_1$ and $IgG_2$ responses following IEB immunisation were to *Cowdria* antigens and not to endothelial cell antigens which contaminate IEB preparations, Western botting was performed. Sera collected at day 28 from an I/T goat (G74) reacted strongly with EB antigens of molecular masses of 21kDa, 24kDa 28kDa 32kDa and others of higher molecular masses which reacted weakly (Figure 5.7 lanes: 2 and 3). These reactions were however only with $IgG_1$ but not to $IgG_2$ (Figure 5.8, lanes 4 and 5).

The $IgG_1$ and $IgG_2$ responses of sera collected on day 34 PI (pre-challenge) from an IEB goat (G668, group C2) were the same as those of the I/T goat (Figure 5.7, lanes: 8 and 9). The very significant $IgG_2$ response after challenge of IEB immunised goats was confirmed by the presence of reactions to *Cowdria* antigens (Figure 5.8, lanes 4, to 6).

### *5.3.4. IgG1 and IgG2 responses of immunised sheep determined by ELISA.*

Infection of sheep with live organisms by blood resulted in a predominant $IgG_1$ response and an apparent absence $IgG_2$ response (Appendix B Table 19a). They were similar to those observed to live infection of goats I/T goats (section 5.3.8).

The IgG responses of sheep (group S2) immunised with recombinant 58kDa Hsp were also characterised by a predominant $IgG_1$ response with a ratio of at least 2:1(Appendix B Table 17) with a non detection of $IgG_2$ in 3 out of 6 animals.

### *5.3.5. $IgG_1$ and $IgG_2$ responses of cattle to live and inactivated antigens of C. ruminantium.*

***IgG1 and IgG2 responses following live infection by experiment (I/T) or by field exposure.***

The *Cowdria* specific $IgG_1$ and $IgG_2$ responses to experimental infection of 7 cattle (group B1) with 3 different stocks of *C. ruminantium* were characterised by a dominant $IgG_1$ but, in contrast to sheep and goats specific $IgG_2$ was detectable above background. The $IgG_1$:$IgG_2$ ratio had a median of 5:1 (range 1:1 to 8:1, Table 5.3).

The $IgG_1$:$IgG_2$ ratios of field sera from S.Africa (group B2) were characterised by $IgG_1$:$IgG_2$ ratios greater than 1 (Table 5.4) with a range from 3:1 to 56:1 (mean 5:1). except for one sample (B200) which had a ratio of 1:2. These ratios were similar to those obtained following experimental infection (Table 5.4) although higher $IgG_2$ levels were observed with 3 sera than observed for any of the animals after experimental infection.

***$IgG_1$ and $IgG_2$ responses following immunisation with IEBs.***

In contrast to goats both $IgG_1$ and $IgG_2$ were detected in sera following immunisation of cattle with IEB's of *C. ruminantium* (Figure 5.9). In addition, the level of $IgG_2$ was much higher after immunisation with IEBs than that obtained in cases of the experimental infection and field sera (Figure 5.9, Table 5.5). Also in contrast to goats the IgG1:IgG2 ratio did not significantly alter after live infection. The $IgG_1$:$IgG_2$ ratios in serum samples of three immunised cattle (S295, S296, S304) were the same pre-challenge and post challenge (1), although there was an increase in OD values of both isotypes in serum after challenge. In contrast to goats the increase in OD value of the two IgG isotypes was of a similar magnitude (Figures 5.9), and did not show greater increase in $IgG_2$. The mean OD values of sera obtained before immunisation of the immunised group and those from two control cattle (S234, S264) remained low and their $IgG_1$:$IgG_2$ ratios were same or $IgG_1$ ratio was higher than 1 (Table 5.5). The control animals did not develop an antibody response prior to death (Table 5.5).

### *5.3.6. $IgG_1$ and $IgG_2$ responses of mice to C. ruminantium after immunisation with I/T, with IEBs or recombinant antigens.*

Immunisation of mice with live *C. ruminantium* was observed to result in a dominant $IgG_2$ isotype response, in contrast to that obtained in live infection of ruminants. Sera obtained on day 34 PI from 4 mice infected with live EBs had higher $IgG_2$ OD values in comparison to sera from mice inoculated with IEBs and collected at day 34 after immunisation (Figure 5.10). The $IgG_1$:$IgG_2$ ratios of the sera from I/T mice was 1:2 and that of sera obtained from the inactivated EB

immunisation was 2:1. The antibody responses of mice immunised with recombinant 58kDa Hsp were observed to be predominantly of $IgG_1$ (Figure 5.11) isotype in specific response, as determined using the 35kDa GroEL antigen.

## *5.4 Discussion*

Immunisation of goats by infection/treatment or with inactivated elementary bodies of *C. ruminantium* led to the development of antibodies to between 7 and 15 EB antigens. The antibodies obtained by immunisation using the two methods detected the same antigenic components of EBs. In contrast, after immunisation with detergent extracted soluble antigens of *C. ruminantium,* antibodies which detected three or four antigenic components developed. This suggests that detergents either destroyed some B cell epitopes or altered the antigenic structure of certain EB components. It may also indicate that detergents are efficient only in extracting four antigenic components of EBs. Alternatively the presentation of soluble antigens may be less efficient at inducing antibody production.

Immunisation with EBs generated antibodies to components of *C. ruminantium* which have been referred to as major antigenic components, their molecular masses being given as 24kDa, 27kDa, 28kDa, 32kDa, 45kDa, 58kDa, and 66kDa in previous studies (Mahan, 1995). Sera from goats immunised with IEB at the CTVM produced antibodies specific to five EB antigens by day 14 post inoculation with molecular masses of 24kDa, 27kDa, 28kDa, 32Da, and 58kDa. This indicates that these antigens are important targets for antibody responses. Furthermore, three of them (28kDa, 32kDa, and 58kDa) are surface antigens as determined by biotin labelling (Section 4.3.2).

Sera obtained after challenge of immunised goats detected more antigens than those obtained before challenge. The appearance of additional antigens after

challenge indicates that these antigens are either expressed by live *C. ruminantium*, or that live infection leads to more efficient antigen presentation. Sera obtained 18 months and 2 years after the last challenge reacted strongly with more antigens of the EB which indicates that a carrier status may have developed after recovery. Carrier status in ruminants which recover from heartwater has been reported in cattle, sheep and African buffalo (Andrew *et al.*, 1989b) and in Creole cattle and goats (Camus, 1992). Furthermore persistence of antibodies for a long period after recovery of ruminants following experimental infection of heartwater has been observed (Semu *et al.*, 1992).

Immunisation of goats with inactivated EBs led to the development of stronger $IgG_1$ than $IgG_2$ responses pre-challenge. After challenge the ELISA OD values of both isotypes were 7 and 10 times those of pre-challenge levels. The increase in $IgG_2$ titres after challenge was such that in two of four goats which survived challenge, the $IgG_2$ OD values were higher than those of $IgG_1$, indicating that this isotype was being preferentially produced. The shift in antibody isotype from $IgG_1$ to $IgG_2$ or an increase in $IgG_2$ after challenge indicated that a Th1 type response was stimulated by the challenge infection. During a Th1 type response $CD4^+$ lymphocytes secrete the cytokines IFN-$\gamma$, IL-2 and TNF-$\alpha$, promoting cell-mediated immunity and $IgG_2$ production. These findings are in agreement with the evidence that stimulation of bovine B cells sorted for IgM with IFN-$\gamma$ induces secretion of $IgG_2$ *in vitro* (Estes *et al.*, 1994). In contrast immunisation of cattle with IEB stimulated production of both $IgG_1$ and $IgG_2$, and challenge of the immunised

cattle led to a further increase in the titres of both isotypes. The differences in IgG isotype responses in goats and cattle to *C. ruminantium* may be associated with the greater resistance of cattle to heartwater (Uilenberg, 1983; Camus and Barre, 1988; Camus *et al.*, 1996).

Sera from two I/T goats (Group 4, G69, G74) infected with the Welgevonden stock and treated to prevent death, had higher levels of $IgG_1$ responses. The reason for these exceptions is not clear. However it suggests that *C. ruminantium* may be inducing production of IL-4 or IL-10 at the beginning of infection which drives the immune responses to a Th2 type antibody responses rather than a Th1 type $IgG_2$/cell mediated immune response.

Cattle immunised by I/T (group 1) and field sera (group 2) had dominant $IgG_1$ responses. It is possible that live infection and immunisation by I/T does not lead to a classical Th1 response but to an IL-4/IL-10 driven response since bovine IL-4 upregulates production of $IgG_1$, IgM and IgGE in the presence of a variety of costimulators (Estes, *et al.*, 1995). Immunisation of cattle with IEBs induced a strong $IgG_2$ response in addition to $IgG_1$, which suggests that a mixed Th1 type and Th2 type response was induced by killed bacteria in contrast to live, or natural (tick mediated) infection. The results suggest live *Cowdria* shifts responses towards Th2. The fact that natural infection and I/T may lead to an IL-4 driven Th2 responses indicates that *C. ruminantium* may avoid the host immune system by directing it towards a Th2 T cell response. However, I/T leads to solid immunity which indicates that other effector mechanisms such as cell-mediated immune responses

mediated by cytotoxic T cell, IFN-γ and NK cells are probably involved in protective immunity. Other workers have found that cytotoxic T cell populations are induced by *Cowdria* infection but only at a suprisingly late stage (Ben Said, Personal Communication Section 6.3.6). This supports the idea that CMI responses are delayed in live infection, an observation made in Section 6.3.6.

The $IgG_1$ response of mice immunised with inactivated EBs was characterised by higher titres of $IgG_1$ whereas that of I/T mice had higher $IgG_2$ titres. These findings are in agreement with those of Du Plessis *et al.* (1991, 1992) who showed that following I/T immunity is cell mediated and that $CD8^+(Lyt2^+)$ T cells were responsible for protection as indicated by adoptive transfer of immune cells to unimmunised mice. The situation in ruminants appears different in that natural infections and I/T leads to a Th2 type response characterised by a dominant $IgG_1$ response.

The role of antibodies in immunity to heartwater is not clear. Experimental transfer of serum or gamma globulins by *in vivo* or *in vitro* neutralisation tests have given variable results (Du Plessis, 1993a; Uilenberg, 1983). Du Plessis *et al.* (1984) found no correlation between antibody titres and immunity to heartwater in calves. Futhermore Martinez *et al.* (1993b) observed that sera from survivors of a challenge experiment after immunisation did not neutralise *C. ruminantium* infection of endothelial cell cultures *in vitro*. However the same authors observed that serum from immune mice and cattle inhibited adhesion and entry of endothelial cell cultures by *C. ruminantium.* In other experiments, Byrom *et al.,* (1993) used mouse serum

with or without complement or purified antibodies administered simultaneously into mice with *C. ruminantium* during incubation or clinical reaction. They observed each treatment did confer immunity or alter the course of *C. ruminantium* infection in mice. In contrast addition of complement to immune serum which was subsequently mixed with infectious *C. ruminantium* (Kumm) inhibited their infectivity (Du Plessis 1993a). The Kumm stock however appears to have a tropism for macrophages and this may be responsible for the different results.

Antibodies are known to be protective in some rickettsial infections. Antibodies enhanced opsonisation and destruction of rickettsiae by neutrophils and macrophages (Gambril and Wisseman, 1973). Experimental transfer of immune sera from mice which had recovered from infections of *E. risticii* prevented disease in 22 out of 24 recipients and purified IgG from immune serum protected mice from infection whereas IgG from non immune mice did not (Kaylor *et al.,* 1991). Further investigation with purified $IgG_2$ may clarify the role played by antibodies in heartwater immunity. Since macrophages and neutrophils have receptors for $IgG_2$ but not $IgG_1$ this may enhance uptake and presentation of *Cowdria.* Further, in *Chlamydia* antibodies to surface MOMP affect intracellular fate, increasing phagolysosome fusion and reducing infectivity (Friis, 1972).

Live immunisation by infection and treatment leads to development of very low $IgG_2$ antibodies in the ruminant whereas it leads to development of a high amounts in the mouse. This indicates that the two species respond differently to live infections of *C. ruminantium* and calls into question the use mice to study immune responses to *C. ruminantium.*

Table 5.1 Antigens of the *Cowdria* EB detected by antibodies in sera from goats immunised with IEB (groups C1 and C2) and those immunised with soluble antigens (group C3) after immunisation and after virulent challenge.

| Antigens detected and Relative mass (kDa) | groups C1+C2 (n= 6) day 34 PI | groups C1+C2 (n= 6) day 28 PC | group C3 (n=3) day 34 PI |
|---|---|---|---|
| 66 | 2+ | 4+ | - |
| 58 | 6+** | 4+ | - |
| 45 | - | 4+ | - |
| 39 | - | 4+ | - |
| 36 | - | 4+ | - |
| 31 | 6+ | 4+ | 3+ |
| 27 | 6+ | 4+ | 3+ |
| 24 | 6+ | 4+ | 3+ |
| 21 | - | 4+ | - |
| 14.5 | - | 4+ | - |

**Key**

** weak reactions with sera of two animals that did not survive challenge

Numbers indicate animals in groups whose sera contained detectable antibodies to *Cowdria* antigens.

Table 5.2. The $IgG_1$ and $IgG_2$ responses and ratios of $IgG:IgG_2$ antibodies in sera from goats immunised with IEBs (gps C1 & C2) after immunisation (day 34 PI) and after challenge (day 28 PC) with virulent blood stabilate of *C. ruminantium*. ELISA absorbance values given are the mean of duplicate tests.

absorbance values at 450nm and ratios of isotypes and day serum was collected.

| | Day 34 PI | | | Day 28 PC | | |
|---|---|---|---|---|---|---|
| | | | Ratios | | | Ratios |
| Goat Nos. | $IgG_1$ | $IgG_2$ | $IgG_1:IgG_2$ | $IgG_1$ | $IgG_2$ | $IgG_1:IgG_2$ |
| G476 | 0.28 | 0.06 | 5:1 | 0.97 | 0.42 | 2:1 |
| G601 | 0.21 | 0.142 | 2:1 | 0.845 | 1.067 | 1:1 |
| G614 | 0.232 | 0.10 | 2:1 | 0.625 | 1.031 | 1:2 |
| G668 | 0.11 | 0.059 | 2:1 | 0.69 | 0.42 | 2:1 |

**Key**
PI = post immunisation
PC = post challenge
Absorbance values given are the mean of 2 tests.

Table 5.3. The mean ELISA absorbance values of *Cowdria* specific $IgG_1$ and $IgG_2$ of antibodies and $IgG_1$:$IgG_2$ ratios in sera from South African cattle immunised by infection and treatment method (gp B1).

| Sample/immuning No/stock used | Mean absorbance at 450nm of isotypes $IgG_1$ | $IgG_2$ | $IgG_1$:$IgG_2$ ratios |
|---|---|---|---|
| B9/Ball 3 | 0.580 | 0.068 | 8:1 |
| B155/Ball 3 | 0.237 | 0.200 | 1:1 |
| B10/Kwanyanga | 0.747 | 0.146 | 5:1 |
| B25/Mara | 0.324 | 0.018 | 18:1 |
| B61/Mara | 0.302 | 0.134 | 2:1 |

**Key**
Absorbance values given are the means of two tests

Table 5.4. The mean ELISA absorbance values of *Cowdria* specific $IgG_1$ and $IgG_2$ antibodies and ratios $IgG_1$:$IgG_2$ in sera collected from naturally challenged cattle in South Africa (gp B2).

| | Mean ELISA absorbance values at 450nm of isotype | | |
|---|---|---|---|
| Sample No. | $IgG_1$ | $IgG_2$ | $IgG_1$:$IgG_2$ ratios |
| B196 | 0.122 | 0.338 | 3:1 |
| B199 | 1.746 | 0.529 | 3:1 |
| B201 | 1.627 | 0.426 | 4:1 |
| B202 | 0.829 | 0.057 | 14:1 |
| B203 | 0.765 | 0.126 | 6:1 |
| B204 | 0.449 | 0.008 | 56:1 |
| B205 | 0.945 | 0.076 | 12:1 |
| B206 | 0.193 | 0.062 | 3:1 |
| B211 | 0.327 | 0.031 | 10:1 |

**Key**
Absorbance values given are the means of two tests.

Table 5.5. The mean ELISA absorbance values of *Cowdria* specific IgG1 and IgG2 antibodies and ratios between the isotype responses in sera collected post immunisation (day 36 PI) and post challenge (day 31 PC) from cattle immunised with IEBs (gpB 3) of *C. ruminantium* and challenge with live Gardel stock.

| | | Mean ELISA absorbance values at 450nm | | |
|---|---|---|---|---|
| Animal No. | Days serum was collected | $IgG_1$ | $IgG_2$ | ratios of $IgG_1$:$IgG_2$ . |
| immunised | | | | |
| S295 | -1 | 0.044 | 0.005 | NA |
| | 36 PI. | 1.319 | 0.881 | 2:1 |
| | 31 P.C. | 1.554 | 1.149 | 1:1 |
| S296 | -1 | 0.022 | 0.004 | NA |
| | 36 P.I. | 0.889 | 0.714 | 1:1 |
| | 31 P.C. | 1.347 | 1.304 | 1:1 |
| S304 | -1 | 0.013 | 0.006 | NA |
| | 36 P.I. | 1.116 | 0.995 | 1:1 |
| | 31 P.C. | 1.347 | 1.260 | 1:1 |
| control infections | | | | |
| S234 C1 | 0 | 0.014 | 0.016 | NA |
| | 11 | 0.035 | 0.016 | NA |
| S264 C2 | 0 | 0.036 | 0.039 | NA |
| | 11 | 0.014 | 0.022 | NA |
| positive control serum B149 | | 0.609 | 0.044 | 14:1 |
| negative B87 | | 0.038 | 0.015 | NA |

**Key**
PC = post challenge
PI = post inoculation
Absorbance values given are the means of two tests.
NA= not applicable.

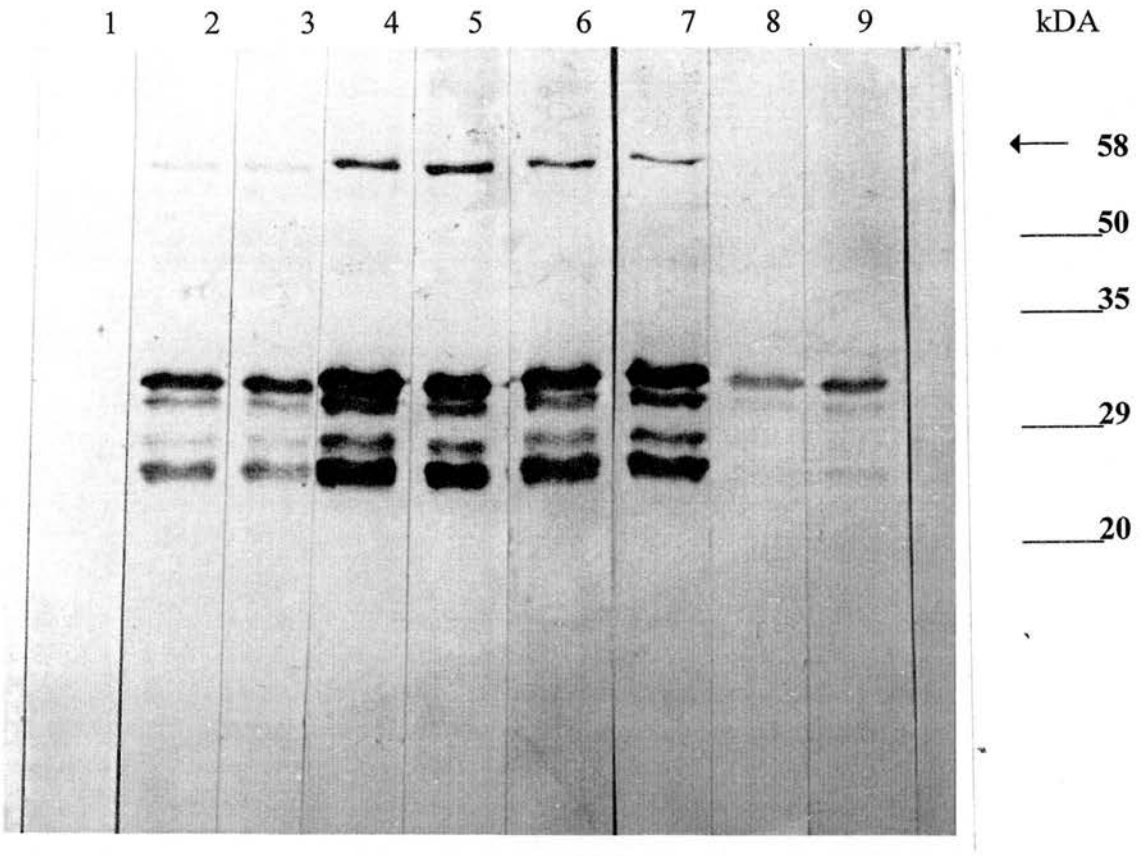



Figure 5.1. Western blots showing antigens of *C. ruminantium* EB detected by antibodies in sera from goats after immunisation infection/treatment (I/T), or with inactivated EBs (IEB) or with detergent extracted soluble antigens (Sags).

The lanes were tested with representative sera from different immunisation regimes as follows: lane 1, negative control serum (G106); lanes 2 and 3, day 28 PI serum from I/T goat (G69); lanes 4 and 5, day 34 PI serum from group1 (IEB); lanes 6 and 7, day 34 PI serum from group C2 (IEB) and lanes 8 and 9, day 34 PI serum from group C3 serum (soluble antigens/Sags).

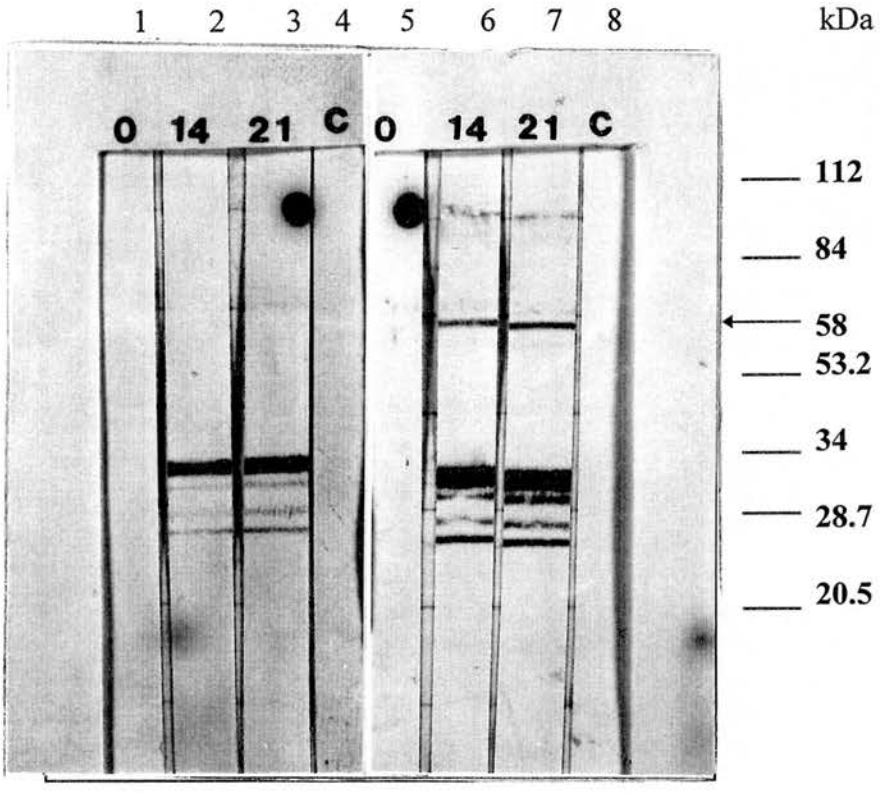



Figure 5.2. Western blots of *C. ruminantium* EB's showing antigens detected by sera from animals after immunisation with inactivated elementary bodies (IEB's).

Lanes 1 to 3 were tested with sera from IEB goat G107; lane 1, day 0; lane 2, day 14 PI, lane 3, day 21 PI; lane 4, negative control (G106)
Lanes 5-7 were tested with sera from IEB goat G108; lane 5, day 0; lane 6, day 14 PI; lane 7, day 21 PI and lane 8, negative control serum (G106).
The figure shows that antibodies were detectable by day 14 PI in both goats.

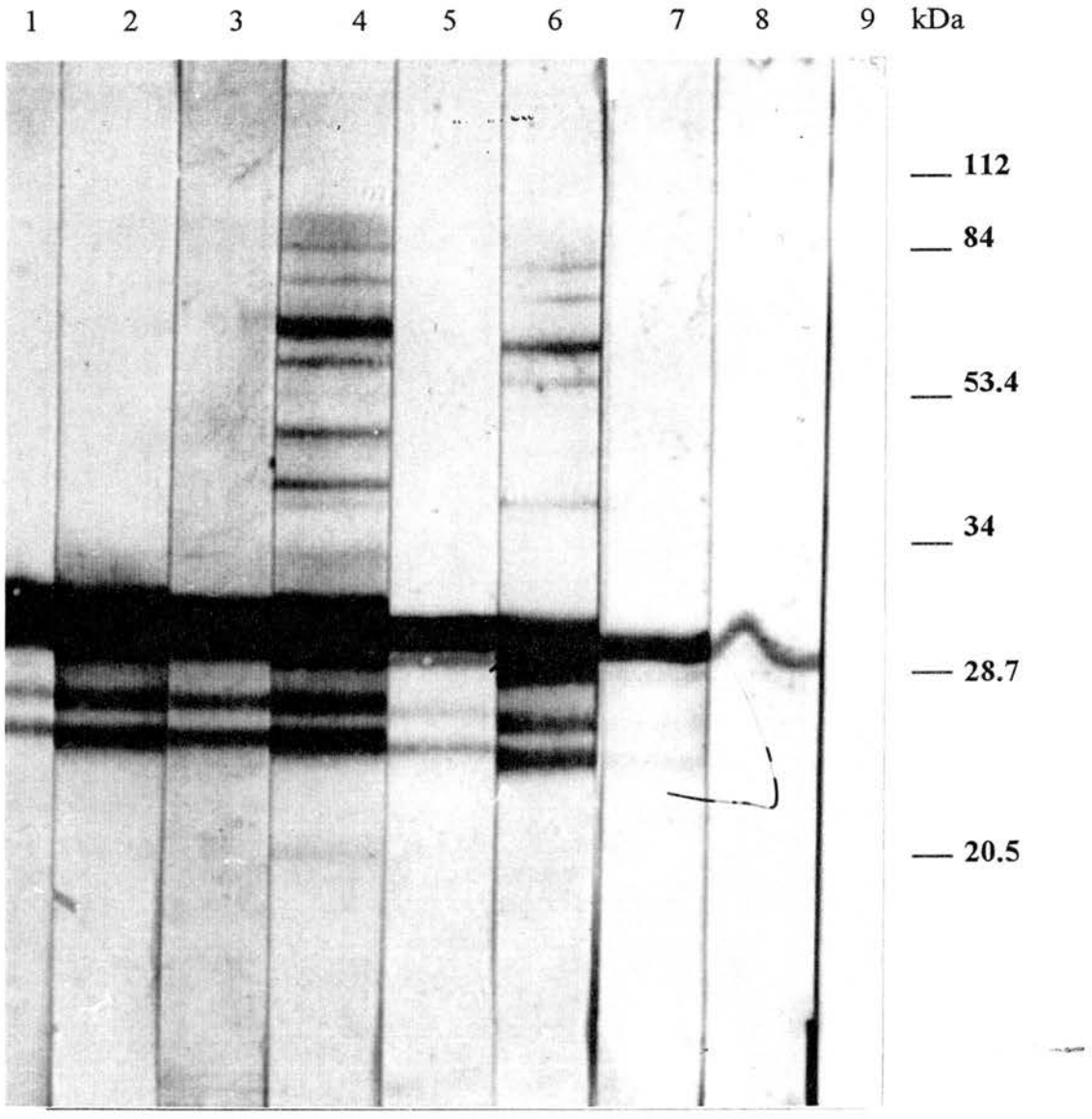



Figure 5.3. Western blots showing antigens of *C.ruminantium* EB recognised by post immunisation (PI) and post-challenge (PC) sera from goats immunised with IEB or soluble antigens (Sags).

Lanes 1 to 3, were tested with day 34 PI sera from an IEB goat (group C1, G476); lane 4, day 28 PC serum (G476); lane 5, post immunisation sera from an IEB goat (group C2, G668), lane 6, day 28 PC serum (group C2 G668), lanes 7 and 8, post immunisation from Sags goat (group C3, G600). Lane 9, negative serum (G106).

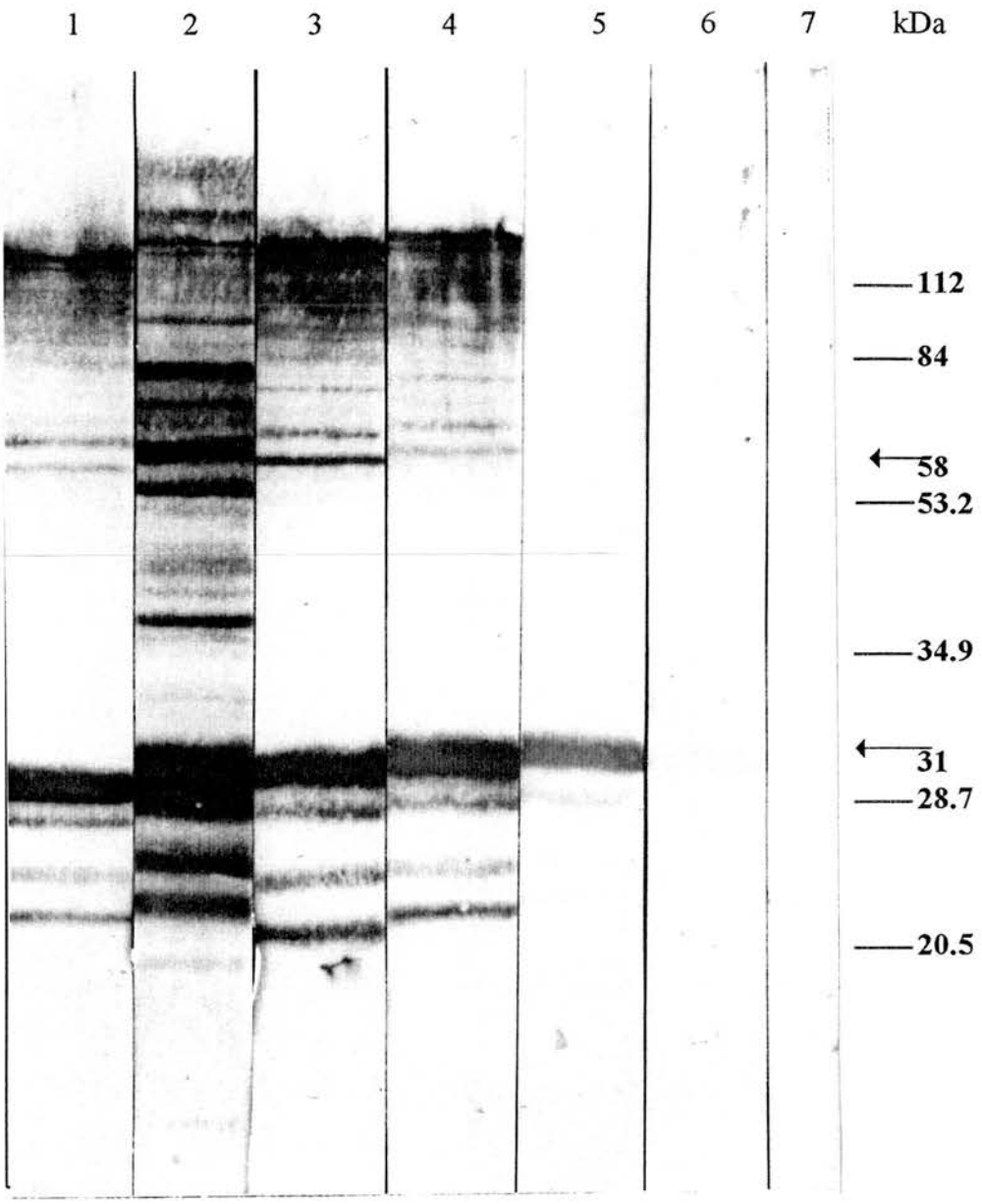



Figure. 5.4. Western blots showing antigens of *C. ruminantium* EB recognised by antibodies following live virulent challenge of IEB immunised goats.

Lanes 1 to 4 were tested with sera from day 28 post challenge of goats in groups C1 and C2 as follows: lane 1, goat 476; lane 2, G614; lane 3, goat G604 and lane 4, G668 Lanes 5 and 6, were tested with with day 57 PI sera from animals immunised with soluble antigens (group C3) and lane 7, tested with sera from negative control.

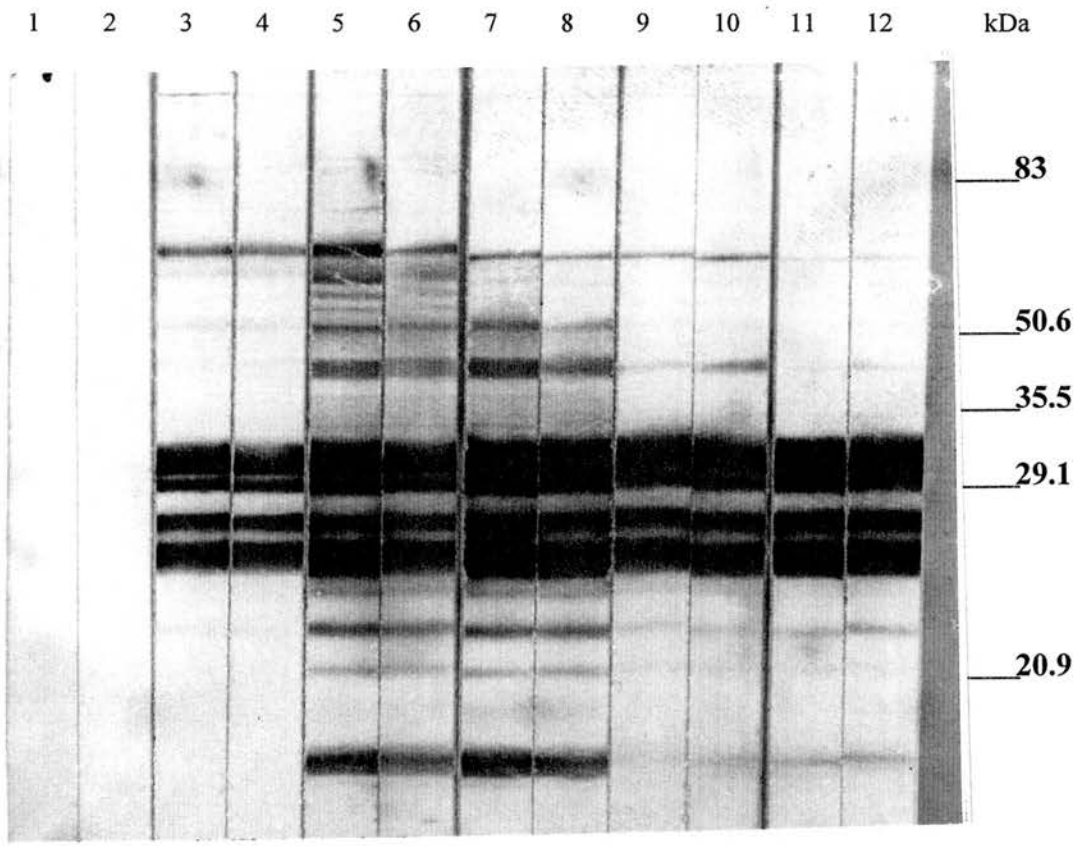



Figure 5.5. Western blots showing reations of goat sera with *C. ruminantium* EB upto 2 years after challenge of IEB immunised goats (G614 and 668).

The lanes were tested with sera as follows: lanes 1 and 2, sera from a naive control goat (G106); lanes 3 and 4, day 28 PI sera from an I/T goat (G74); lanes 5 and 6, sera obtained 18mo PC from G614, lane 7 and 8, sera obtained 18mo PC from G668, lanes 9 and 10, sera obtained 24mo PC from G614; lane 11 and 12, sera obtained 24 mo from (G668). Note the strong reactions with several antigens of 18mo sera compared to reactions of day 28 sera from I/T goat (G74) used as positive control.

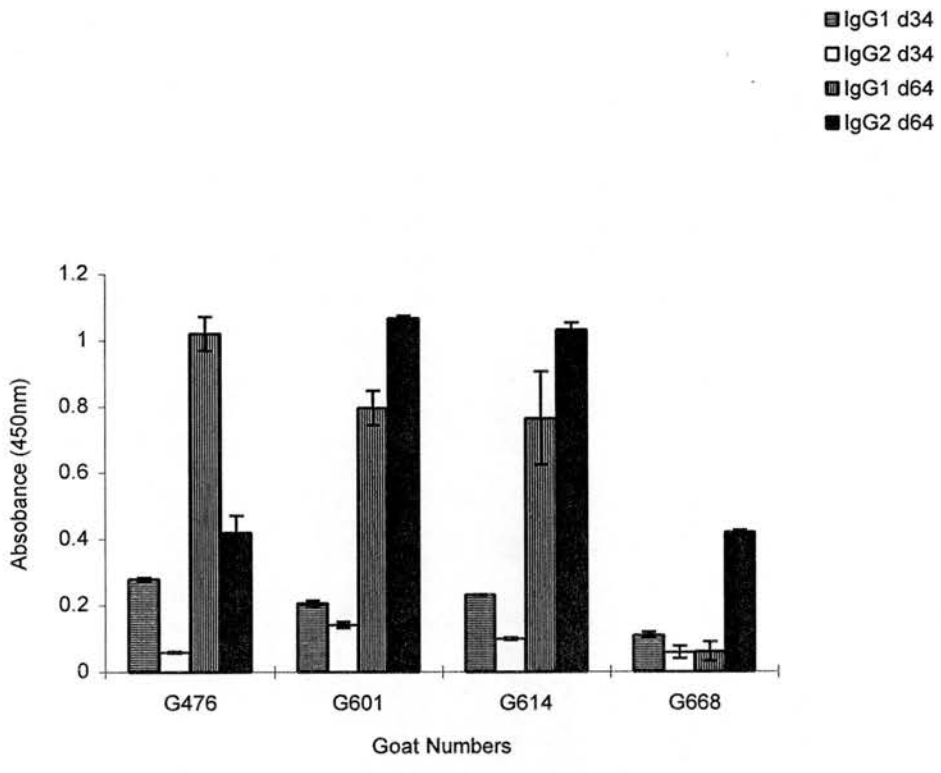



Figure. 5.6. The post immunisation (PI) and post challenge (PC) $IgG_1$ and $IgG_2$ responses of 4 goats immunised with IEB of *C. ruminantium* detected by ELISA. Post immunisation and post-challenge sera were collected day 34 PI and day 28 PC (=Day 64 PI) from each animal.
The bars show the mean OD of 2 tests and the error bars show the Standard error of the mean.

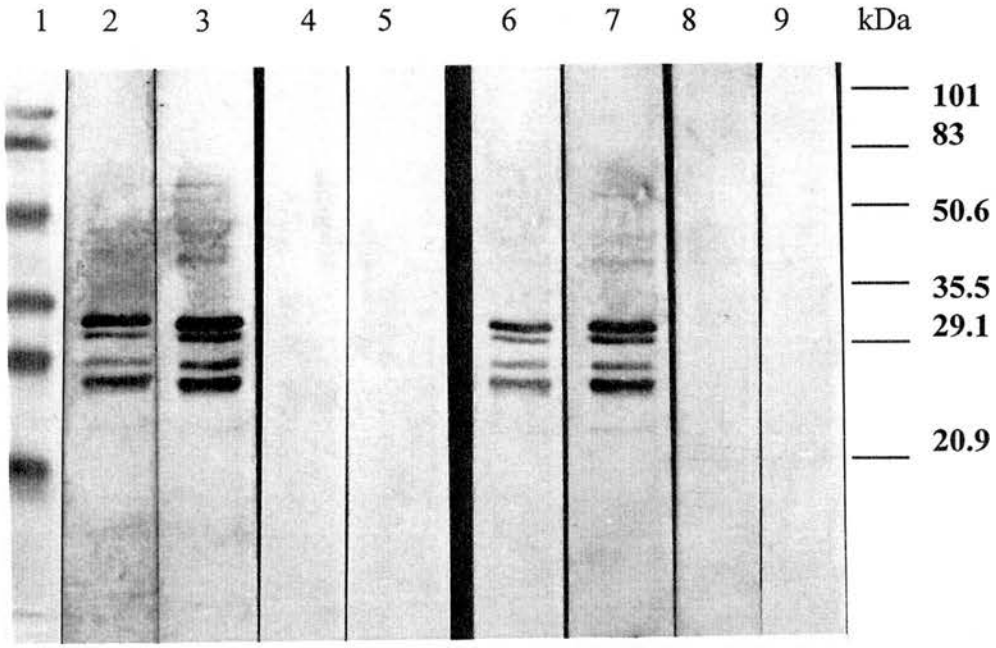



Figure 5.7. Evidence of specific $IgG_1$ response against *Cowdria* antigens following immunisation by infection/treatment and by IEB immunisation.

Lanes: 1 and 10, SDS low range molecular weight protein standards;
Western blots were reacted with sera in duplicate and then tested with anti-$IgG_1$ or anti-$IgG_2$ monoclonal antibodies. Lanes 2 to 5, day 28 PI sera from an I/T goat (G 74) lanes 6 to 9, day 34 PI serum from IEB goat (G668).
Lanes 2, 3, 6 and 7 were reacted with anti-$IgG_1$ Mab; lanes 4 and 5 with anti-$IgG_2$
Note the lack of detectable IgG2 following I/T or IEB immunisation.

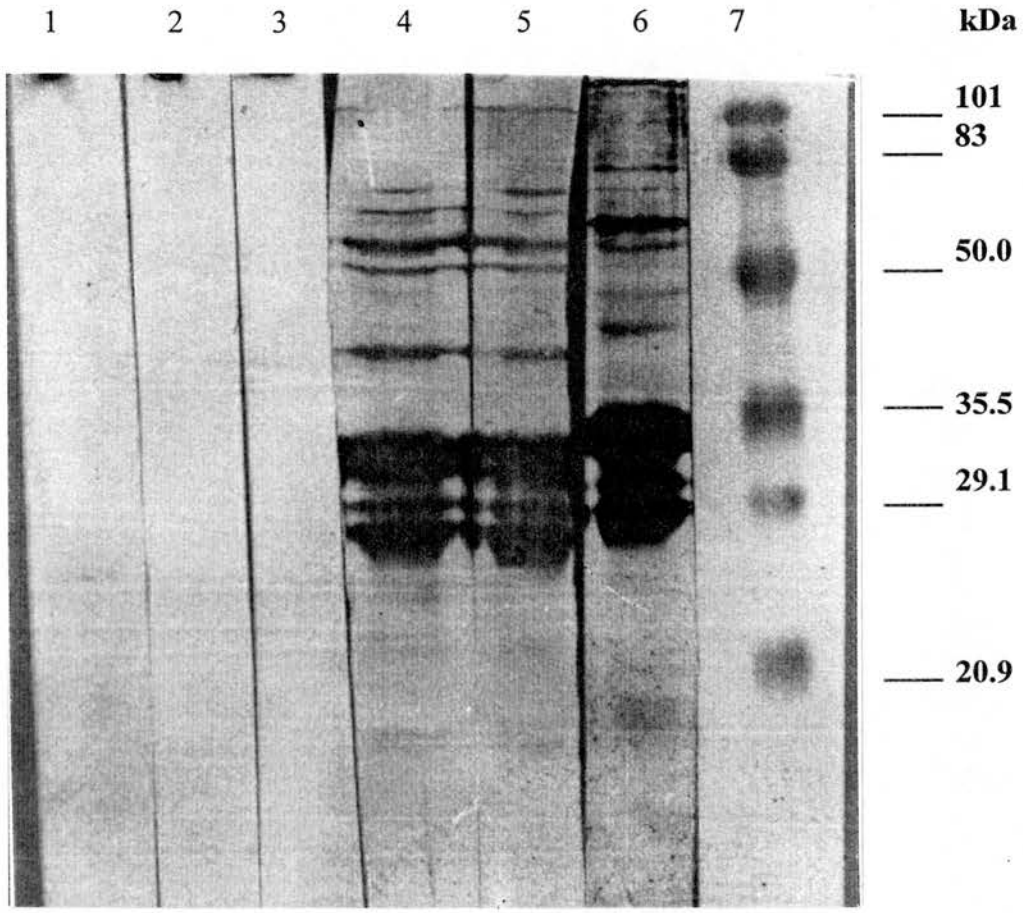



Figure 5.8. Evidence for *Cowdria* specific IgG2 responses following challenge of IEB immunised goats with virulent homologous stock of *C. ruminantium*.
Lanes 1 to 3, negative control serum (G106); lanes 4 to 6, serum collected day 28 post challenge of IEB immunised goat (G476); lane 7 SDS protein standards.

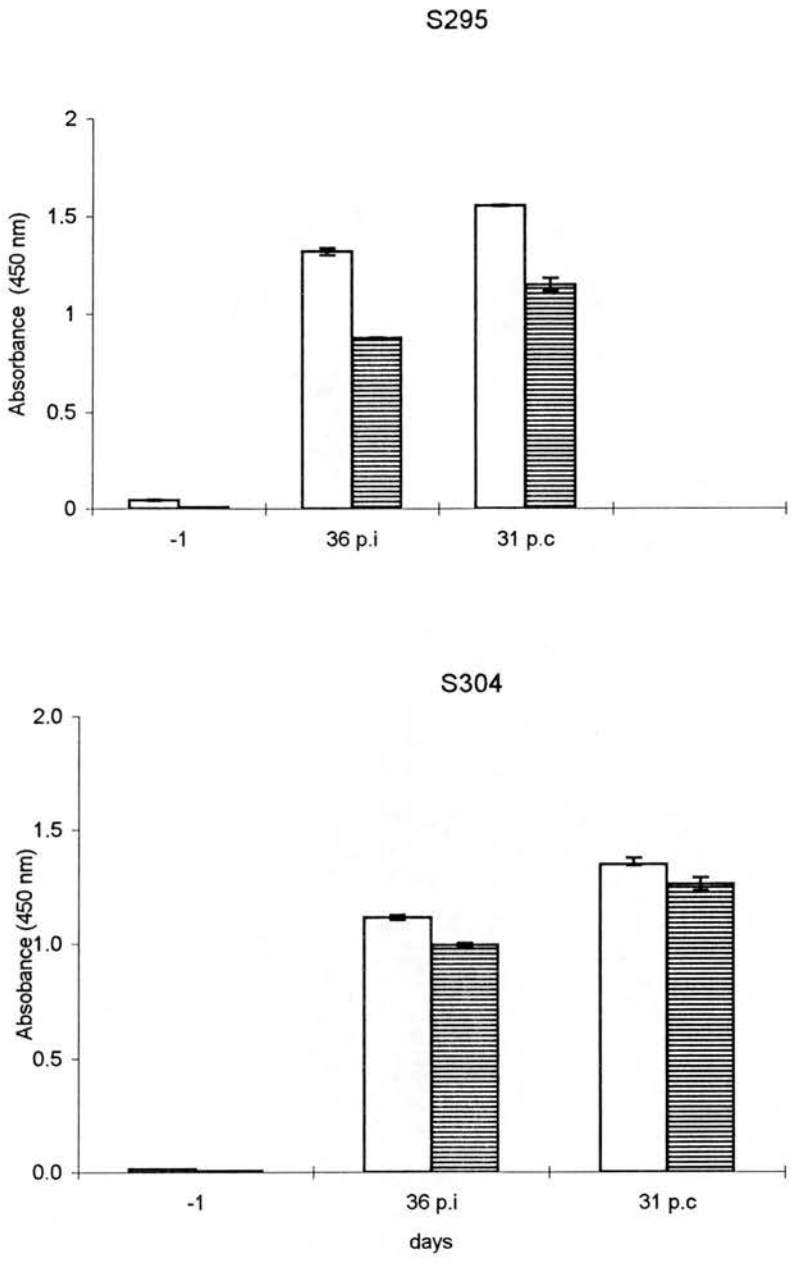



Figure 5.9. $IgG_1$ and $IgG_2$ responses of pre-challenge (day 36 PI) and post-challenge(day 31 PC) sera of two Kenyan cattle (S295, S296) immunised with IEBs (gp B3) of *C. ruminantium* (Gardel).
IgG1responses ( ▭ ) andIgG2 responses ( ▤ ).
Bars represent the mean ELISA OD 450 of two tests of sera collected 36 days

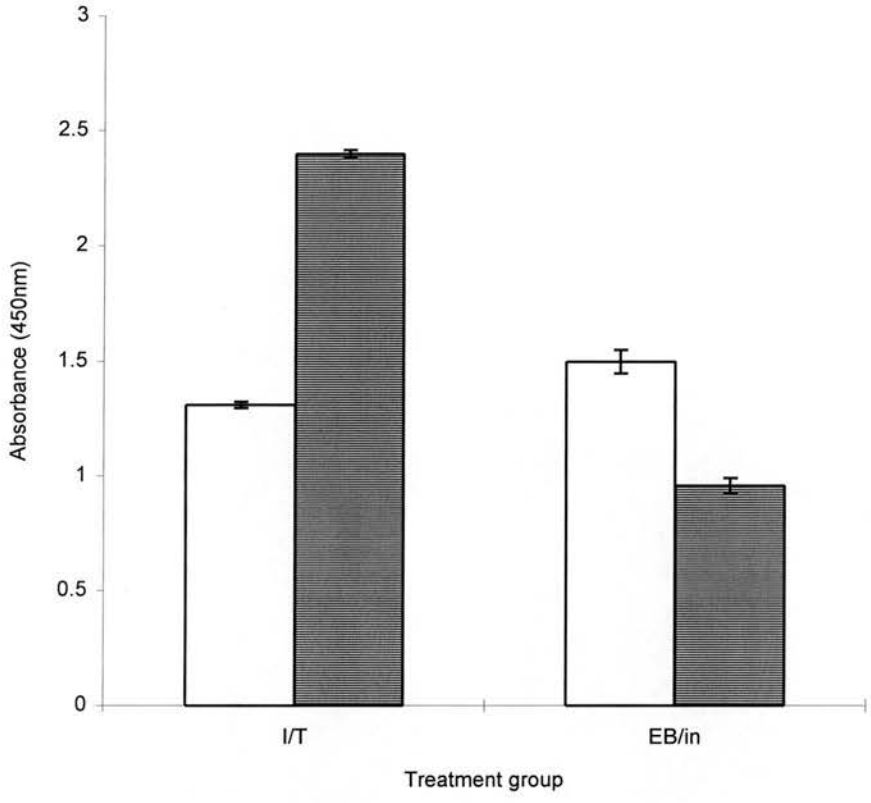



Figure 5.10. The $IgG_1$ and $IgG_2$ responses of mice immunised by I/T or with IEB of *C. ruminantium*. Sera were collected 34 days after infection or immunisation, pooled and tested for IgG1 and IgG2. IgG1responses (□) and IgG2 responses (▒). Results are expressed as the mean OD 450nm of 2 tests. Error bars show standard error of the mean.

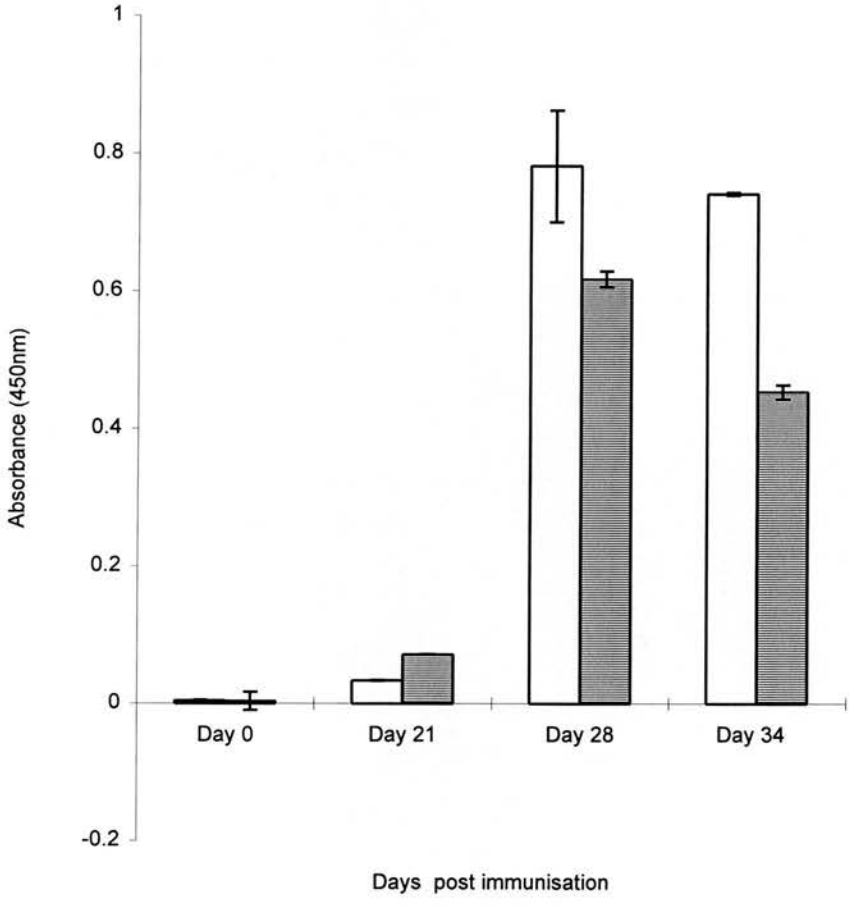



Figure 5.11. $IgG_1$ and $IgG_2$ response ofsequential sera from mice (10) immunised with recombinant 58-kDa Hspantigen of *C. ruminantium* at days 21, 28 and 34 after immunisation.
IgG1responses ( □ ) and IgG2 responses ( ▩ ) were detected by ELISA.
Bars represent the mean of two tests and the error bars show the standard error of the mean.

# CHAPTER SIX

# *IN VITRO* AND *IN VIVO* RESPONSES OF LYMPHOCYTES FROM GOATS IMMUNISED BY INFECTION AND TREATMENT OR WITH INACTIVATED ELEMENTARY BODIES TO *C. RUMINANTIUM*

## *6.1. Introduction*

Animals which recover from natural and experimental *C. ruminantium* infection are solidly immune to homologous challenge and show a variable degree of resistance to heterologous challenge (Oberem and Bezuidenhout, 1987a). Animals which are immunised with inactivated organisms are immune to homologous challenge (Martinez *et al.*, 1994; Mahan *et al.*, 1995). The specific mechanisms responsible for protective immunity in ruminants to *C. ruminantium* are not clearly understood (Du Plessis, 1993; Uilenberg and Camus 1993; Camus *et al.,* 1996). However, the relative roles of antibodies were investigated (Section 5.3.7 & 5.3.9) and there was an indication that antibodies ($IgG_2$) may be important in protective immunity. In mice immunity is mediated by cytotoxic Lyt-$2^+$ T cells (Du Plessis *et al.*, 1991; Du Plessis *et al.,* 1992).

*C. ruminantium* is an obligate intracellular bacterium found in membrane bound vacuoles in the cytoplasm of endothelial cells (Cowdry, 1925a, 1926; Prozesky and Du Plessis, 1987) and neutrophils (Logan *et al.,* 1987b). It is partially protected from the direct effects of antibody by its intracellular location. Immunity to intracellular bacteria is mediated by cellular immune responses that are generated following antigen presentation by professional and non-professional phagocytes

(Kaufmann, 1993). The specific cell types which carry out antigen presentation *in vivo* include macrophages/monocytes, dendritic cells, B cells and gut epithelial cells (Grencis, 1990). Antigen capture by antigen presenting cells (APC) is facilitated by cell surface receptors which include Fc receptors and immunoglobulins (Lanzavecchia, 1996). Intracellular pathogenic organisms adhere to, enter or invade macrophages situated in the skin, gastro-intestinal tract and lungs or in the liver and spleen if the organism is blood-borne. Phagocytosed bacteria undergo degradation in the phagolysosome by proteases resulting in the generation of immunogenic fragments (peptides) that form a complex with MHC class II molecules. The peptides are then expressed on the surface of the APC and presented to T cell receptors (TCR) on the surface of T lymphocytes (Ojcius *et al.*, 1996). The TCR is a heterodimer of alpha/beta glycoprotein chains on $CD4^+$ and $CD8^+$ T cells (Fitch, 1986). T cells play a key role in determining the direction of the immune response by secreting various cytokines. The $CD4^+$ T-helper cells are capable of two distinct responses: T-helper 1 (Th1) and T-helper 2 (Th2). $CD4^+$ T cells are very important in the development of acquired immune resistance to pathogenic bacteria (Hahn and Kaufmann, 1981). The $CD4^+$ Th1 T cells play a key role in immune responses to intracellular bacteria by secreting cytokines such as IFN-$\gamma$ which activate mononuclear phagocytes into potent effector cells. IFN-$\gamma$ is also produced by other mediators of acquired resistance such as $CD8^+$ T cells, $\gamma/\delta$ T-cells and natural killer (NK) cells (Kaufmann, 1993). Th2 T cells are responsible for antibody-mediated responses which are driven by interleukin-4 (IL-4) and interleukin-10 (IL-10). $CD8^+$

T cells recognise antigen presented by MHC class I antigens and effect cytotoxic activities on virally infected cells and those infected by some protozoan parasites (Howard and Morrison, 1994). Certain microbial components trigger the release of cytokines such as TNF-α and IL-12 from macrophages and other professional phagocytes (Cheers and Zhan, 1996). These two cytokines have several protective effects enhancing non-specific anti-microbial activity. TNF-α and IL-12 cause NK cells to release IFN-γ which promotes microbicidal activity of macrophages and neutrophils, causes changes in endothelial cells and phagocytes which results in greater adhesion of phagocytes to blood vessel wall thereby facilitating entry of the phagocytes into sites of inflammation (Delanoy *et al.*, 1993). IFN-γ cause $CD4^+$ T cells to differentiate into Th1 type cells which effect cell mediated immune responses and supports $IgG_2a$ production in mice (Mosmann and Coffman, 1989).

In view of the fact that protective immunity against *C. ruminantium* in mice is mediated by Lyt-$2^+$/$CD8^+$ T cells, I attempted to characterise the role of cellular responses of ruminants in protective immunity to *C. ruminantium*.

The aim of experiments in this chapter was to investigate T cell mediated responses of goats immunised with live EBs or with inactivated elementary bodies (IEBs) of *C. ruminantium* by studying:

1. The blastogenic responses to EBs of peripheral blood mononuclear cells (PBMC) from immunised animals.
2. Changes in lymphocyte sub-populations in peripheral blood of immunised animals following stimulation with EBs *in vitro*.

3. Changes in lymphocyte populations in the peripheral blood of immunised animals following challenge with virulent homologous stock of *C. ruminantium*.

## 6.2. Materials and Methods

### *6.2.1. Antigens*

Inactivated elementary bodies (EBs) of three stocks of *C. ruminantium* were used. The stocks were: Ball 3 (Haig, 1952), Gardel, (Jongejan *et al.,* 1984) and Welgevonden (Du Plessis, 1985a). They were propagated in bovine endothelial cell cultures as described by Pow *et al.* (1993), then 10-14 day old cultures were harvested by centrifugation as described in Section 3.4. The EBs were washed, re-suspended in sterile PBS and inactivated with 0.15% formalin for 30 minutes at room temperature. Protein was determined by the Bicinchoninic acid assay (BCA, Protein Assay kit, Pierce, USA) as described in Section 3.13 and five dilutions of inactivated EBs (1µg, 5µg, 10µg 15µg and 20µg/ml) were prepared in complete RPMI 1640 medium and used to stimulate PBMC.

### *6.2.2. Experimental animals*

Six British Saanen goats aged 18 to 24 months old were used in these experiments. They belonged to three groups of two goats each; infection and treatment immunised goats (G69, G74) described in Section 4.2.2, goats immunised with inactivated elementary bodies (G107, G108) described in Section 3.14.1 and naive controls (G109, G110).

### *6.2.3. Isolation, culture and in vitro stimulation of peripheral blood mononuclear cells (PBMC) from goats with C. ruminantium antigens.*

PBMC from the six goats described above (6.2.2) were used for cell proliferation assays or flow cytometric analysis. The PBMC were collected from the different immunisation groups on the same occasion (being day 14, and 21 PC for the I/T goats and day 28, 48 and 143 PI for the IEB goats). The IEB goats received boosters then 7 days later PBMC were taken for proliferative tests. PBMC were prepared and counted as described in Section 3.2.

One millilitre stock cultures of PBMC containing 2 x $10^6$ cells were prepared from each goat, then antigen or mitogen was added respectively.

In order to determine which test was to be used for subsequent *in vitro* cell proliferative responses, stocks of PBMC cultures from the two I/T goats (G69 and G74) were stimulated with 1, 5, 10, 15 or 20 μg/ml of inactivated homologous EB (Welgevonden stock) antigen in duplicate. Unstimulated controls from each animal were included. The cultures in aliquts of 200 μl in 96 well sterile tissue culture plates (Nunc, Denmark) and incubated as described in Section 6.2.4 then, one set of cultures was used to determine proliferative responses by blastogenesis and the other set by MTS assay.

***Experiment 1.***

The aim of this experiment was to determine the effect of mitogen (Concanavalin), homologous (Welgevonden) or heterologous EB antigens (Ball 3, Gardel) of *C. ruminantium* on the *in vitro* proliferative responses of PBMC from two goats immunised with inactivated EBs (G107 and G108) and a control goat (G109).

PBMC cultures were stimulated with 5 μg/ml of the mitogen Concanvalin A, or with 1, 5, 10, 15 or 20 μg/ml of inactivated EBs. Unstimulated control cultures were included. Four tests were performed and their median responses were used.

***Experiment 2.***

The aim of this experiment was to compare the proliferative responses of PBMC from goats immunised by I/T (G74) or those immunised with IEB (G107, G108) following *in vitro* stimulation with 10 μg/ml of homologous EBs or 5 μg/ml of mitogen Concanavalin A. PBMC were obtained 21 PC from the I/T goat at day 28 PI from the IEB goats purified as described earlier (Section 3.2) and stimulated with respective stimulant.

***Experiment 3.***

This experiment was carried out to determine if SDS-PAGE purified EB antigens of 24kDa and 32kDa (Section 4.2.6) or purified 35kDa recombinant antigen could stimulate proliferation of PBMC from immune goats. PBMC was collected from the on day 21 PC from two I/T goats (G69 & G74) and from a naive control (G109) purified as described earlier and stimulated *in vitro* with 1 or 5 μg/ml of the SDS-PAGE EB antigens, 2.5μg/ml or 5 μg/ml of a purified 35kDa recombinant subclone protein antigen (Section 3.7). The responses of these cultures were determined by counting blasts using cytospins.

For determination of IFN-γ produced *in vitro* by stimulated cultures, PBMC from the I/T goats (G69, G74) on day 21 PC, IEB goats (G107, and G108) day 28 PI and one naive control goat (G110). After purification they were stimulated with 15 μg/ml of

EB protein of the homologous stock (Welgevonden), or 5 μg/ml of recombinant 35kDa subclone protein (Section 3.7) of *C. ruminantium*. The cultures were incubated as described earlier (Section 3.2) for 72 hours. The PBMC from these cultures were used for flow cytometric analysis to determine any changes in the proportions of $CD4^+$ or $CD8^+$ after stimulation.

### *6.2.4. Cell proliferation assays*

After 72 hours, 100 μl of culture supernatant was withdrawn from each well and replaced with fresh 100 μl of medium, the cultures were then incubated for a further 48 hours under the same conditions. Proliferation was detected by a metabolic assay using the Cell Titre 96® Non-Radioactive MTS assay (Promega Madison, USA) or by blastogenesis. The MTS assay detects the enzymatic activity of mitochondrial dehydrogenases produced by dividing cells on the tetrazolium salt (MTS/Owens reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), which is reduced into coloured formazan salts soluble in tissue culture medium and which can be measured spectroscopically. Twenty microlitres of the MTS with 5% v/v of solubising solution (Phenazine methasulfate/PMS) was added to each well and the plates incubated for 4 hours to allow solubilisation. The absorbance (optical density/OD) 570nm was taken after incubation using a plate reader (Multiscan Plus, Labsystems). The corrected median OD values were calculated by subtracting the median OD values of unstimulatedcultures from the median OD value of test cultures. The corrected ODs were used to plot dose response curves.

The proliferative responses determined by blastogenesis were carried out by examination of cytospin slides stained with Giemsa. The cytospin slides were prepared by taking 100 µl aliquots from three cultures, cytospining them onto slides, fixing in methanol for 5 minutes and staining them for 40 minutes in 5% Giemsa. They were washed in tap water, dried in air and examined at 400x magnification for lymphoblasts using a Labofluor microscope (Leitz). The number of lymphoblasts per 200 cells examined on each slide were determined and the means three slides was calculated. The corrected mean cuonts used for the were obtained by subtracting number of lymhoblasts in unstimulatedcontrol cultures these means were transformed into percentages and used in the results.

### *6.2.5. Determination of the effect of live or inactivated EBs on the proliferative responses of PBMC from an infection/treatment goat (G74).*

A preliminary study was carried out to determine the effect of live or inactivated EBs on the proliferative responses of PBMC collected 21 days PC from one I/T goat (G74) *in vitro.*

EBs were prepared as described in Section 3.4, half of the washed EBs was inactivated and the other half was left untreated. Serial ten-fold dilutions were carried out on each EB fraction (to give 190 µg/ml, 19 µg/ml, 1.9 µg/ml and 0.19 µ g/ml EB protein) and used to stimulate PBMC collected day 28 PC. The cultures were incubated as described in Section 6.2.4, and their proliferative responses were determined by the MTS assay or by examination of Giemsa-stained cytospins as described in Section 6.2.4. The mean of three tests was calculated and the effect of

antigen concentrations was analysed by the paired student t-test using only results obtained by MTS.

### *6.2.6. Detection of gamma interferon (IFN-γ) in culture supernatants*

Assays for IFN-γ were carried out using supernatants taken from 72 hour cultures and preserved at -70°C (Section 6.2.4).

IFN-γ detection was carried out using a commercial capture ELISA kit for the detection of bovine IFN-γ (Bioscience, Australia) following the manufacturer's instructions. Supernatants from unstimulatedcultures or cultures stimulated with 5 μg/ml of Concanavalin A, 15 μg/ ml of EB (Welgevonden stock) or 5 μg/ml of recombinant 35kDa protein were tested in triplicate, using 50 μl aliquots of supernatant per well. Supernatants from PBMC of control goats were included in each assay. The absorbance (OD) was read at 450nm using a plate reader (Multiscan Plus, Labsystems) and the mean absorbance values of three tests was calculated. The corrected mean OD values of IFN-γ were obtained by subtracting the mean IFN-γ OD valueof unstimulated cultures (background) from that of the stimulated cultures. The corrected OD values were used to plot graphs and for statistical analysis using the student t-test.

### *6.2.7. Preparation of lymphocytes after in vitro stimulation with IEBs for flow cytometry using a fluorescence activated cell scanner.*

PBMC from cultures described earlier for determinanation of of IFN-γ production (Section 6.2.3) were used for flow cytometric analysis. Five day cultures were harvested by centrifugation at 350 x g for 6 minutes. The supernatants were

discarded and the cells were washed twice in FACS buffer (PBS containing 0.1% sodium azide, 1% bovine serum or bovine serum albumin and 10u heparin/ml/). Washed cells were suspended in 1 ml of buffer, cell viability and counting were carried out and the cell concentration adjusted to $1x10^6$ cells/ml as described earlier (Section 3.2). Two hundred microlitres of each sample were aliquoted into three labelled tubes. Fifty microlitres of undiluted mouse monoclonal antibodies to ovine T-cell surface antigens ($CD4^+$, $CD8^+$ and γδ) were added to each tube. Monoclonal antibodies to T cell subsets (Table 6.1) were obtained as culture supernatants from Dr. John Hopkins, Dept. Veterinary Pathology, Royal (Dick) Scho ol of Veterinary Studies, Edinburgh.

Following incubation at room temperature for 30 minutes the cells were washed twice with FACS buffer. Labelled cells were stained by the addition of 50 µl of a 1/1000 dilution of sheep anti-mouse fluorescein conjugated antibodies (Table 6.1, Binding Site, Birmingham, UK) to the respective isotype antibodies prepared in FACS buffer. The cells were incubated for 30 minutes in the dark at 4°C washed and 10,000 cells were analysed by flow cytometry as described by Woldehiwet and Sharma (1990) using a FACScan (Becton Dickinson, Cowley, UK), gating for mononuclear cells.

### *6.2.8. Challenge of immunised and control goats.*

Five goats (one I/T, 2 IEB, and 2 controls) challenged with a virulent Welgevonden stock of *Cowdria ruminantium* as described earlier (Section 3.16). Goats were placed in isolation unit of the CTVM before challenge and on the day of

challenge, (day 7 after the last booster for the IEB goats and 3 months PC for the (I/T) each goat was inoculated with 3 x $10^5$ EBs intravenously through the jugular vein in 2 ml of culture medium. The EBs used for challenge were propagated and counted as described earlier (Section 3.4 and 3.15 respectively).

#### *6.2.8.1. Sample collection and monitoring of goats during challenge.*

Prior to and after challenge, two blood samples were taken from each goat, one was used for routine haematology and the other for isolation of lymphocytes for flow cytometry. Haematology samples were collected in EDTA and used to determine total white blood cell count (WBC) and differential white cell counts. Total WBC was determined using an automated hematology analyser (Biochem Immunosystems, Allentown, PA, USA). Differential counts were determined by preparation of blood smears, staining with modified Wright's stain, using a Hematex 2000 slide stainer (Bayer Diagnostics, UK) and the cells were counted using a standard reference technique. The haematology samples were analysed at the Haematology laboratory of the Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, Edinburgh. Further blood sampling was carried out from each goat on days 3, 7 and 10 post challenge and additional blood samples were taken from the I/T goat (G74) on days 17, 21 and 32 post challenge. PBMC for flow cytometric analysis were prepared and stained for $CD4^+$, $CD8^+$ and γδ TCR positive cells using the monoclonal antibodies and FITC conjugates shown on Table 6.1. The proportion of positive T lymphocytes for each surface antigen were transformed into absolute numbers using the total and differential counts as described

by Woldehiwet and Sharma (1990). The absolute numbers of T cells were used to determine $CD4^+$ and $CD8^+$ ratios. Animals which developed severe clinical signs were euthanised by intravenous administration of pentobarbitone. Rectal temperatures were recorded daily for 5 days before challenge until the end of the experiment. Confirmation of heartwater was carried out by making brain crush smears from dying goats as described by Purchase (1945).

## *6.3. Results*

### *6.3.1. Proliferative responses of PBMC of immunised goats to inactivated EBs*

PBMC from I/T goats (G69 and G74) proliferated in a dose-dependent manner in the presence of homologous EB antigens of the Welgevonden stock (Figure 6.1a and b). This dose response was observed by the MTS assay and examination of cytospins. The MTS assay was used for majority of cell proliferation responses hereafter.

***Experiment 1***

PBMC from I/T or IEB goats responded in a dose-dependent manner to EBs from the homologous (Welgevonden) and heterologous (Ball 3 and Gardel) stocks. Lower concentrations of EB antigens (5 μg/ml and 1 μg/ml for G107 and G108 respectively) of the homologous (Welgevonden) stock produced larger transformation response (Figure 6.2a and b) and higher concentrations of antigen were inhibitory (Figures 6.2a and b). Twenty micrograms per millilitre of EB antigens of the Ball 3 stock stimulated the highest responses. Responses to the Gardel stock were greatest at 5 μg/ml and 20 μg/ml of EB for G108 and G107 respectively (Figure 6.2a, 2b). Proliferative responses of PBMC from the control goat (G109) were much lower than those of the IEB goats (Figure 6.2c.), although they had a similar pattern of dose responses.

***Experiment 2***

Stimulation of PBMC obtained from the I/T goat (G74), or IEB goats (G107 and G108) with 10 μg/ml of Welgevonden EBs or 5 μg/ml of Concanavalin A

showed that the three mmunised goats responded to EBs whereas the control did not (Figure 6.3). The proliferative responses of the I/T goat (G74) and IEB goat (G108) were similar but those of IEB goat (G107) were lower. Interestingly EB had an inhibitory effect on proliferative responses from control goat (G110) (Figure 6.3). PBMC from all the animals proliferated in the presence of Concanavalin A with the highest OD values being shown by PBMC from the negative control goat (G110).

***Experiment 3.***

PBMC obtained from two I/T (G69 & G74) did not proliferate in the presence of SDS-PAGE purified antigens of molecular masses 24kDa and 32kDa of the homologous stock (Welgevonden). Similarly PBMC from immune goats did not proliferate in the presence of 2.5 or 5 μg/ml of purified 35kDa subclone protein but they proliferated in the presence of the mitogen Concanavalin A and homologous EBs (Figure 6.4).

### 6.3.2. *Live elementary bodies lowered the proliferative responses of PBMC from an Infection/treatment (I/T) goat.*

Stimulation of PBMC from I/T goat (G74) with live or inactivated elementary bodies showed that live EBs stimulated lower responses than inactivated EBs (Figure 6.5a and b). However there were no significant difference between the responses stimulated by the two preaparations ($t = 1.02$, $p > 0.005$). The OD values of cultures stimulated with 190 μg/ml and 19 μg/ml of IEB protein were higher than those stimulated with the same concentrations of live EBs in the MTS proliferative the assays (Figure 6.5a). The same effect was observed the blastogenic responses

although cultures stimulated with 0.19 μg/ml protein of inactivated elementary bodies stimulated the highest proportion of cells.

### *6.3.3. Production of interferon gamma (IFN-γ) by PBMC from I/T and IEB goats stimulated with EBs of C. ruminantium in vitro.*

Homologous EBs stimulated PBMC from 3 of 4 goats immunised by I/T or with IEBs of *C. ruminantium* to secreted IFN-γ *in vitro*. However there were no significant differences between the IFN-γ responses of I/T and the IEB group ($t = 2.38$, $p > 0.05$). The OD values of IFN-γ from PBMC of goats immunised with live *C. ruminantium* were up to 2.6 times higher than those obtained from goats inoculated with inactivated EBs (Figure 6.6 and Table 6.3). The responses varied between individual goats that had received the same treatment. Supernatants from G69 had higher amounts of IFN-γ than those from G74, and within the IEB goats supernatants from G107 had higher amounts of IFN-γ than those from G108. IFN-γ responses of PBMC from the control goats (G110) were very low. Culture supernatants of PBMC from goats stimulated with recombinant 35kDa subclone of GroEL antigen of *C. ruminantium* contained little or no IFN-γ.

### *6.3.4. Effect of in vitro stimulation with EBs of PBMC from immunised goats on the proportion of $CD4^+$ and $CD8^+$ T cells.*

Flow cytometric analysis of lymphocytes following *in vitro* stimulation with EBs showed that $CD4^+$ T cells increased by 1 to 3 percent after stimulation whereas $CD8^+$ T cells decreased by 7% in stimulated cultures (Table 6.2) as compared to unstimulated The CD4:CD8 ratios ranged between 1.2 and 1.4:1 in EB stimulated cultures. In contrast in cultures stimulated with Concanavalin A, the ratios ranged

between 1:1.3 to 1:2. In all the goats Concanavalin A stimulated a higher proprtion of $CD8^+$.

### *6.3.5. Clinical responses of goats to homologous challenge.*

Four of five goats reacted with high fever 8 to 9 days after challenge (Figure 6.8), with the exception of the I/T goat (G74) which did not develop fever or other clinical signs of heartwater. Fever continued until the goats died 3-5 days later showing clinical signs of heartwater. One IEB goat (G108) and one control goat (G110) died suddenly without clinical signs except fever. At post mortem classical pathological changes including hydrothorax, lung oedema and hydropericardium were present in all animals and petechial haemorrhages were present on the mucosa of the abomasum of two goats (G110 and G107). Examination of brain impression smears revealed the presence of abundant *C. ruminantium* in the cytoplasm of endothelial cells of blood capillaries in.

### *6.3.6. Changes in counts of PBMC in the peripheral blood of I/T IEB and control goats after challenge with virulent homologous isolate of C. ruminantium (Welgevonden stock).*

The absolute numbers of $CD4^+$ and $CD8^+$ T cells in peripheral blood of the I/T goat (G74) remained relatively constant up to day 10 PC (Figure 6.8a). On day 17 PC, there was a dramatic rise in the absolute numbers of $CD8^+$ T cells ( Figure 6.8a, Appendix B, Table 2). The CD4:CD8 ratio in the first 10 days of infection was between 2:1 to 1:1 but as a result of the dramatic increase of $CD8^+$ T cells on day 17 PC the ratio between the two T cell types was 1:5 in favour of $CD8^+$ T cells. The $CD8^+$ T cells counts remained higher than those of $CD4^+$ T cells for another one

month although it dropped with time to 1:2 on the last day of analysis. The $CD4^+$ and γδ T cell counts of this goat did not alter significantly from pre-ch allenge counts throughout the challenge period (Figure 6.8a).

The $CD4^+$ and $CD8^+$ counts in peripheral blood of the IEB goats were characterised by a general decrease in cells numbers from pre-challenge counts except those of G107 which increased slightly on day 3 PC (Figure 6.8b, c). The γδ counts remained unchanged after challenge except those of G108 which decreased after day 3 PC and remained about the same until the last day of analysis (Figure 6.8b).

The $CD4^+$ and $CD8^+$ counts in the peripheral blood goats in the control group behaved in a similar manner to those of one IEB goat (G108), they declined from pre-challenge counts after challenge except for the $CD8^+$ counts of G110 which increased on day 7 PC and continued to rise to the highest counts on day 10 PC (Figure 6.8e, d). The high number of $CD8^+$ in peripheral blood of G110 at day 7 PC was surprising, it was most likely to be caused non-specific factors since this was a naive animal. The γδ counts also declined after challenge (Figure 6.8d, e)

Analysis for differences between the $CD4^+$, $CD8^+$ and γδ T cell counts of IEB and those of the controls showed that there were no significant differences between them ($t= 0.67$, $p> 0.05$; $t=-1.70$, $p>0.05$; $t= -1.53$, $p>0.05$).

### *6.3.7. Changes in monocyte and neutrophil counts in peripheral blood of goats immunised with IEBs and controls after challenge with virulent homologous stock.*

The monocyte counts of the I/T goat (G74) increased from pre-challenge counts to the highest numbers on day 7 PC, and remained almost the same until the last day of analysis (Figure 6.9a). The monocyte counts of the IEB goats increased to 4 times pre-challenge counts by day 10 PC. On the other hand the counts of the controls decreased below pre-challenge counts on day 3 PC followed by an increase in numbers attaining the highest counts on day 10 PC (Figure 6.9a). Analysis for group to group differences between the monocyte counts of the IEB and control groups showed no significant differences ($t = 0.94$, $p > 0.05$).

The neutrophil counts of the I/T goat and controls were characterised by fluctuations (Figure 6.9b). Their counts increased above pre-challenge counts on days 3 and 10 PC but decreased on day 7 PC (Figure 6.9b). On the other hand those of the IEB goats increased to the highest counts on day 7 PC, and decreased slightly on day 10 PC (Figure 6.9b). There were however no significant differences between the mean counts of the IEB and controls n=2 revealed ($t = 0.15$, $p => 0.05$).

## 6.4. Discussion.

Elementary body antigens of *C. ruminantium* induced an antigen specific T cell responses in PBMC obtained from goats previously immunised with *C. ruminantium* antigens. Low concentrations of homologous antigens produced the maximum proliferative response whereas high concentrations of heterologous stocks were needed to stimulate maximal responses. These results indicate that differences in the responses to heterologous and homologous antigen occur which, may be an important component of the lack of cross-protection between strains of *Cowdria.* However cross-protection between IEB immunised animals is not yet determined.

The presence of epitopes in heterologous EB antigens which induce responses may indicate the presence of conserved epitopes between strains which stimulate T cell responses these, could be utilised in vaccine development. Antigenic fractions could be investigated to determine antigens which induce similar responses in animals immunised with homologous and heterologous stocks.

Stimulation of PBMC from goats immunised with live or inactivated antigens of *C. ruminantium* with homologous EBs induced similar T cell responses. The proportion of $CD4^+$ slighty increased after *in vitro* stimulation but those of $CD8^+$ decreased suggesting that the EBs stimulated a T helper T cell response and not a T cytotoxic one.

Stimulation of PBMC obtained from the two treatment groups (I/T and IEB immunisation) with homologous EB antigens led to IFN-γ production. This indicates that immunisation by I/T or with IEB primes PBMC to secrete IFN–γ upon *in vitro*

stimulation and suggests that a Th1 type immune response is induced after immunisation. The concentration of IFN-γ in supernatants of PBMC obtained from I/T were 2 times higher than those from IEB immunised goats. The reason for this is unclear but it suggests that live organisms are more potent inducers of IFN-γ production than inactivated EBs. IFN-γ is secreted by $CD4^+$ Th1, $CD8^+$, γδ and natural killer cells *in vivo* (Kaufmann, 1993), and plays a pivotal role in cell-mediated immune responses. The fact that supernatants from PBMC of goats stimulated with EB contained IFN-γ indicates that the induced response was of a Th1 type and not a Th2 type. IFN-γ favours a CMI response by inhibition of IL-4 secreted by Th2 T cells, a cytokine whose effects lead to a Th2 type antibody response (Tizard 1994).

Stimulation of PBMC obtained from an I/T goat (G74) with live antigens lowered the proliferative responses whereas inactivated antigens did not. The reduction of the lymphocyte proliferative responses by live *C. ruminantium* suggests that one of the mechanisms used by this rickettsia to evade the immune response of the host is by down regulation of lymphocyte activation. T cell activation is dependent on antigen association with MHC class II or MHC class I molecules and production of Interleukin I (IL-1) by antigen presenting cells in particular macrophages (Grencis, 1990; Tizard, 1992). Therefore any process which alters macrophage expression of MHC class II molecules or IL-1 production significantly influences the immune response (Splitter and Everlith, 1989). Liposaccharides (LPS) suppresses expression of class II molecules on the surfaces of macrophages

(Koerner, Hamilton and Adams, 1987), inhibit induction of IFN-α and maintenance of class II molecule expression on macrophages (Everlith, Brooksalder and Splitter, 1991). Evidence of reduced lymphocyte proliferative responses to specific antigens caused by the down regulation of MHC class II has been observed with the intracellular bacteria *Brucella abortus* (Splitter and Everlith, 1989) and *C. ruminantium*; (Totte *et al.,* 1996). Other mechanisms used by intracellular bacteria to evade the immune responses of lymphocytes and monocytes/macrophages include:- Induction of hydrogen peroxide release, secretion of prostaglandins and other mediators capable of inhibiting lymphocyte function (Shenker and Slots 1989, Shenker, Vitale and Slots, 1991), selective killing of lymphocytes by toxic products and impairment of lymphocyte activation by interfering with IL-2 receptor expression and reduction of IL-2 production (Shenker, *et al.*, 1987, Shenker, Vitale and Slots, 1991). IL-2 is necessary for activation of B and T lymphocytes and induces the release of other cytokines such as TNF-α and IFN-γ which play a very important role in anti-microbial immunity (Feng, Popov and Walker, 1994). The mechanisms used by *C. ruminantium* to reduce lymphocyte responsiveness and proliferation are unknown. Evidence from *in vitro* studies with bovine endothelial cells infected with *C. ruminantium* indicate that infected endothelial cells do not express MHC class II on their surface (Totte *et al.*, 1996). This report suggests that *C. ruminantium* inhibits expression of MHC class II which in turn will delay development of an effective immune response to this organism.

Immunisation of small ruminants with inactivated EBs of *C. ruminantium* leads to development of protective immunity (Tafesse, 1992; Martinez *et al.*, 1993; Mahan *et al.*, 1995). Tafesse (1992) observed that some immunised goats did not survive challenge with a blood stabilate containing virulent homologous stock. The two IEB goats used in this study were not protected against challenge with virulent homologous stock. There are two possible reasons for this, the IEB inoculation did not induce immunity or the challenge dose given ($3.0 \times 10^5$ EBs/dose) was too high. The latter suggestion is supported by the sudden deaths of two goats and the fact that the Welgevonden stock causes a more severe disease in small stock than the other stocks (Du Plessis *et al.*, 1989).

Lack of protection of immunised goats in this study may also be related to the low INF-γ responses of IEB goats compared to the I/T goat which recovered. IFN-γ is a strong inhibitor *C. ruminantium in vitro* and *in vivo* (Totte *et al.,* 1994, Totte *et al.*, 1996). This cytokine has inhibitory properties against other rickettsial agents *in vitro* for example *Rickettsia prowazekii* (Turco and Winkler, 1983a), *R. tsutsugamushi* Gilliam strain (Hanson, 1991), *Chlamydia psittaci* (Byrne and Krueger, 1983), *Ehrlichia risticii* (Park and Rikihisa, 1991). IFN-γ also has protective properties *in vivo* against *R. conorii* (Li *et al.*, 1987), in recovery from pnuemonia caused by *Chlamydia trachomatis* in mice (Byrne, *et al.*, 1987) and is required for resistance against *Listeria monocytogenes* during early stages of infection (Nakane, *et al.,* 1989) before T-cell mediated immunity develops. Since CTL responses in heartwater appear late in infection of EB immunised animals after

recovery has commenced (Section 6.3.6, Bensaid personal communication 1996) IFN-γ may be critical to early control.

Following challenge the infection/treatment goat (G74) did not develop any clinical symptoms of disease as compared to the IEB inoculated goats. The T-cell responses of this goat did not change significantly for the first 10 days after challenge and the CD4:CD8 ratios was greater than 1. This suggested that other immune effector mechanisms were in operation. The fact that the numbers of $CD4^+$ cells were the same or higher than those of $CD8^+$ cells up to the 10th day post challenge suggests that the T cell response to the challenge was mediated by T helper cells rather than $CD8^+$ T cells. The scenario changed by the 17th day after challenge whereby $CD8^+$ T cells dominated for another month.

The possible mechanisms responsible for protective immunity before a potentially protective $CD8^+$ T cell response was in operation in the I/T goat could have been by INF-γ as *in vitro* stimulation of PBMC from this goat produced higher amounts of INF-γ than those from IEB inoculated goats. IFN-γ has a direct lytic effect on rickettsiae within infected cells (Hanson, 1991) and acts as an important factor in controlling rickettsial infections *in vivo* (Hanson, 1991; Jerrels *et al.*, 1986; Li, *et al.*, 1987; Turco and Winkler, 1983a, b, 1993). IFN-γ has direct effect on the *in vitro* infectivity of *C. ruminantium,* reducing the infectious yield of *C. ruminantium* from bovine endothelial cells (Totte *et al.,* 1994), and also inhibits the growth of *C. ruminantium* in bovine endothelial cells *in vitro* (Mahan *et al.*, 1996). IFN-γ stimulates the anti-microbial activity of macrophages by regulating oxygen

metabolism, and the synthesis of reactive nitrogen intermediaries from arginine (Feng, Popov and Feng, 1994). It also activates professional phagocytes, including neutrophils and natural killer cells to kill invading organisms, it is important in the activation of lymphocytes in response to infection and the up-regulation of MHC class II and class I, leading to effective response to infecting organism.

Neutrophils play a role in cell mediated-immunity by participating in two mechanisms of cytotoxicity namely antibody dependent cell-mediated cytotoxicity (ADCC) and antibody independent cell-mediated cytotoxicty (AIDCC). In ADCC targets are opsonised by $IgG_2$ and IgM. These two classes of antibody act as important opsonins for bovine and ovine neutrophils (Roth, 1994). ADCC is expected to participate in clearing *C. ruminantium* from the body via IgM or $IgG_2$. However $IgG_2$ was not detectable in sera of one I/T goat (G74) after previous challenge but was present in sera of both IEB immunised goats (Chapter 5). Both ADCC and AIDCC are increased by pre-treatment of neutrophils with recombinant IFN-γ *in vivo* and *in vitro* (Lukacs, *et al.* , 1985; Chiang, *et al.* , 1991). It is most likely that goat neutrophils were activated to kill *Cowdria* by AIDCC by IFN γ induced by challenge. Bovine neutrophils have been shown to be active in antibody independent killing (Lukacs *et al.*, 1985; Steinberg *et al.*, 1989). Protective activity of cytolytic $CD4^+$ (Th1), $CD8^+$ and possibly γδ T cells could also account for immunity before the numbers of $CD8^+$ increases. The results suggests that at least 10 days are required for $CD8^+$ T cells to reach levels greater than those of $CD4^+$ T cells. Immunity to heartwater in the infection/treatment animal was probably mediated by multiple effectors, IFN-γ being the major activator before protective $CD8^+$ T cell response developed.

Table 6.1. Monoclonal antibodies and FITC antibodies used for flow cytometric analysis

| Antibody | Specicificity | Isotype | FITC |
|---|---|---|---|
| ST4 | CD4 | $IgG_1$ | Mo-$IgG_1$-FITC |
| 86D | γδ | $IgG_1$ | Mo-$IgG_1$-FITC |
| SBU | CD8 | $IgG_{2a}$ | Mo-$IgG_{2a}$-FITC |

**Key**
FITC = fluorescein isothiocyanate conjugated immunoglobulins

Table 6.2. Proportions of $CD4^+$ and $CD8^+$ T cells of I/T, IEB immunised and control goats after *in vitro* stimulation with 15μg/ml of EB's anntigens of the Welgevonden (homologous) stock of *C. ruminantium*. The CD4:CD8 ratios are shown in the last column.

| | | % of T cells | | |
|---|---|---|---|---|
| Goat Nos/group | stimulants | $CD4^+$ | $CD8^+$ | CD4:CD8 ratios |
| G69 (I/T) | 0 | 37.4 | 15.95 | 2:1 |
| | Con A | 22.85 | 52.70 | 1:2 |
| | EB | 38.00 | 21.32 | 2:1 |
| G74 (I/T) | 0 | 23.83 | 28.00 | 1:2 |
| | Con A | 30.00 | 38.00 | 1:1 |
| | EB | 27.0 | 21.00 | 1:1 |
| G107 (IEB) | 0 | 23.50 | 26.02 | 1:1 |
| | Con A | 9.35 | 14.70 | 1:2 |
| | EB | 26.30 | 16.67 | 2:1 |
| G108 (IEB) | 0 | 53.30 | 12.00 | 4:1 |
| | Con A | 20.00 | 33.00 | 1:1 |
| | EB | 56.10 | 12.5 | 5:1 |
| G110 (control) | 0 | 36.90 | 39.40 | 1:1 |
| | Con A | 23.40 | 44.9 | 1:2 |
| | EB | 38.3 | 24.00 | 2:1 |

Summary of percentage increase or decrease of $CD4^+$ and $CD8^+$ after stimulation

Table 6.3. Prduction of IFN-γ by PBMC of immune goats after *in vitro* stimulation with EBs of *C. ruminantium* or Concanavalin A.

| | Groups and IFN-γ OD values (450 nm) | | | | |
|---|---|---|---|---|---|
| | I/T | | IEB | | Control |
| Stimulators | G69 | G74 | G107 | G108 | G109 |
| EB (15μg/ml) | 0.650 | 0.334 | 0.274 | 0.102 | 0.082 |
| 35 kDa (5μg) | 0.063 | 0.088 | 0.069 | 0.056 | 0.010 |
| ConA (5μg) | 2.300 | 3.04 | NA | 0.250 | 2.880 |

**Key.**

The results given are the mean absorbance values (450nm) for 3 tests.
NA = not applicable samples were not available.

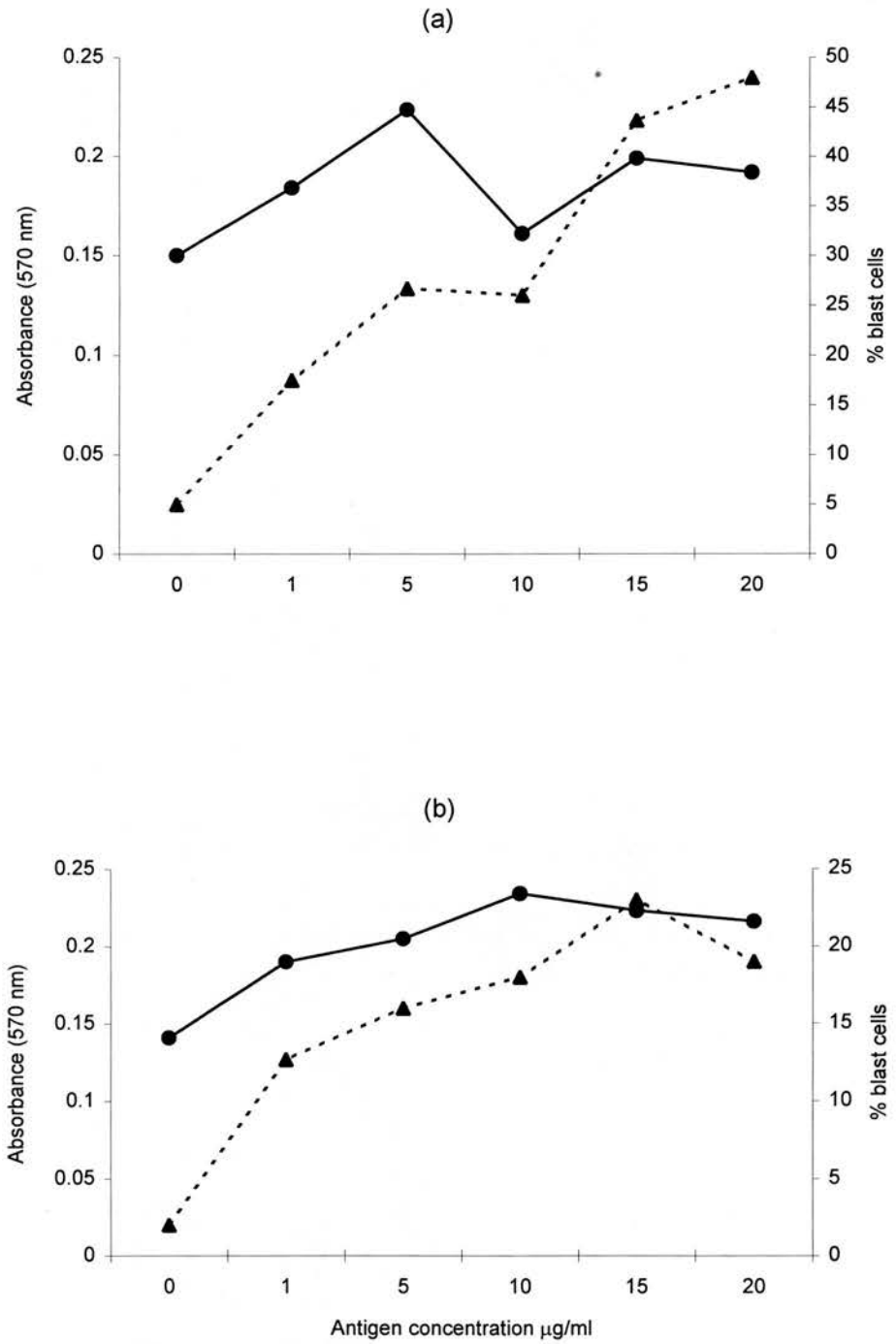



Figure 6.1. Dose-dependent *in vitro* proliferative responses of PBMC from two I/T goats G69 (Figure 6.1a) and G74 (Figure 6.1b) stimulated with homologous EBs (Welgevonden stock) of C. ruminantium.
Level proliferation measured by MTS assay (▲) or by blastogenesis (●)
The results given are medians of three test.

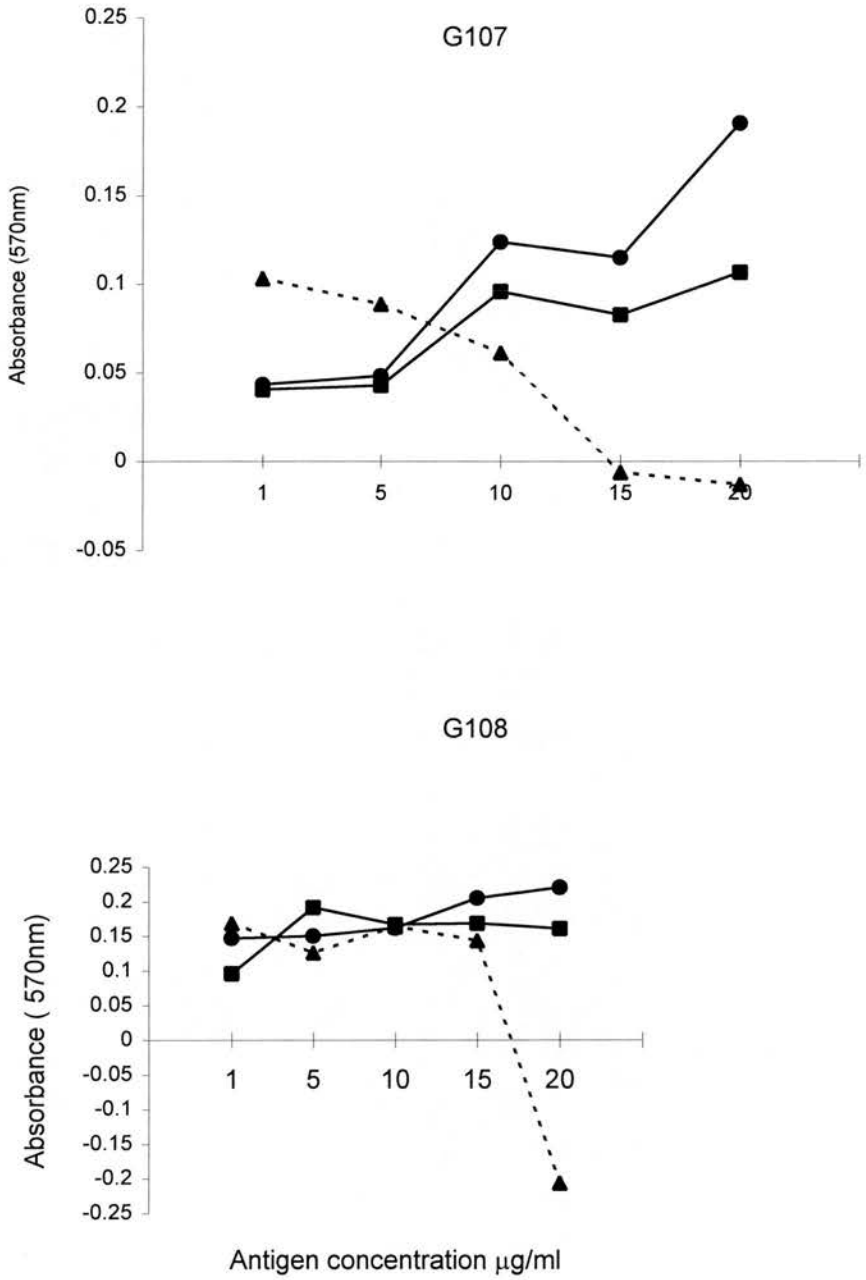



Figure 6.2. Dose-dependent *in vitro* proliferative responses of PBMC from two IEB immunised goats (G107, G108) to EB antigens of homologous (Welgevonden) and heterologous (Ball 3, Gardel) stocks of *C. ruminantium*.
Proliferative responses to Welgevonden ( ▲ ) Ball 3 ( ● ) and Gardel ( ■ ).
The results given are median of 4 tests.

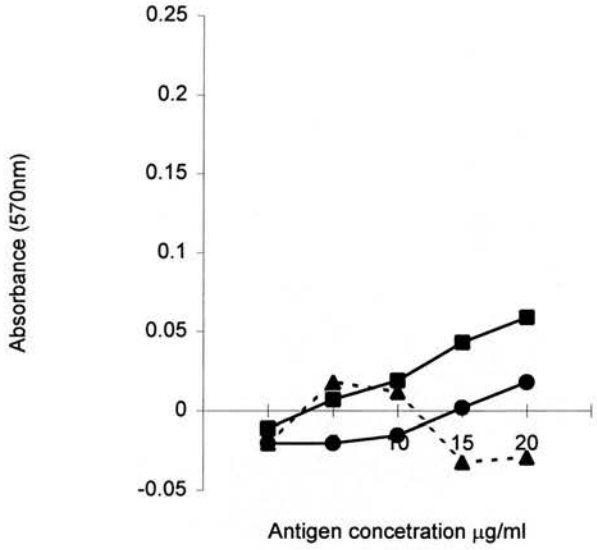



Figure 6.2c. Proliferative responses of PBMC from unimmunised control goat (G109) to EB antigens of the Ball 3, Gardel Welgevonden stocks of *C. ruminantium*. Proliferative responses to Ball 3 (●), Gardel (■) and to Welgevonden (▲). The median ODs of 4 tests are given.

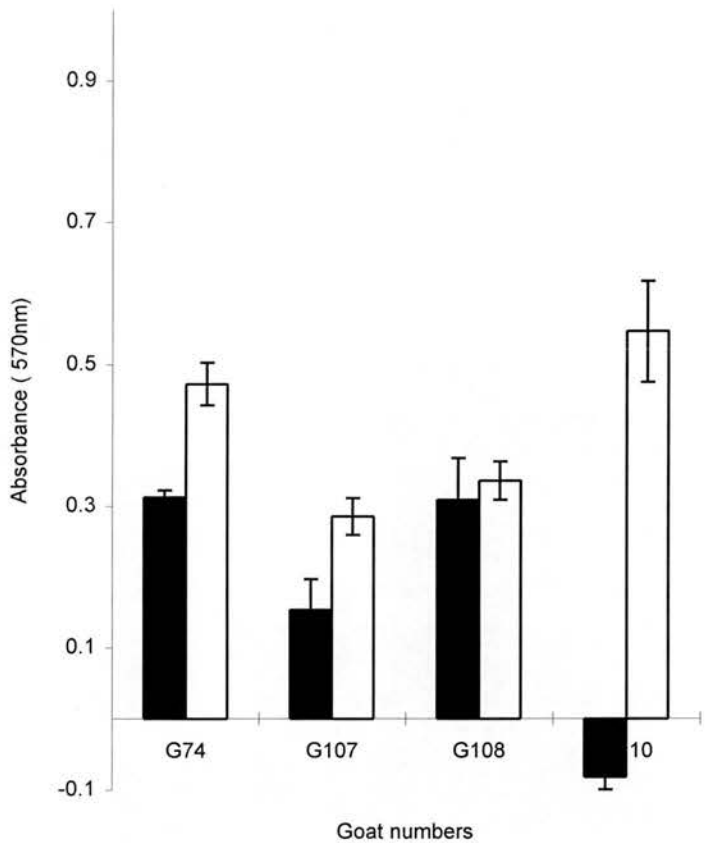



Figure 6.3. Proliferative responses of an I/T goat (G74), IEB goats (G107 & G108) and a naive control after *in vitro* stimulation 15 μg/ml of EB antigen of the Welgevonden stock and or with 5 μg/ml of Concanavalin A.
Proliferative responses to EBs ( □ ), and to Concanavalin A ( ■ )
Proliferative responses were determined by MTS assay and results given are the correccted mean of three tests.

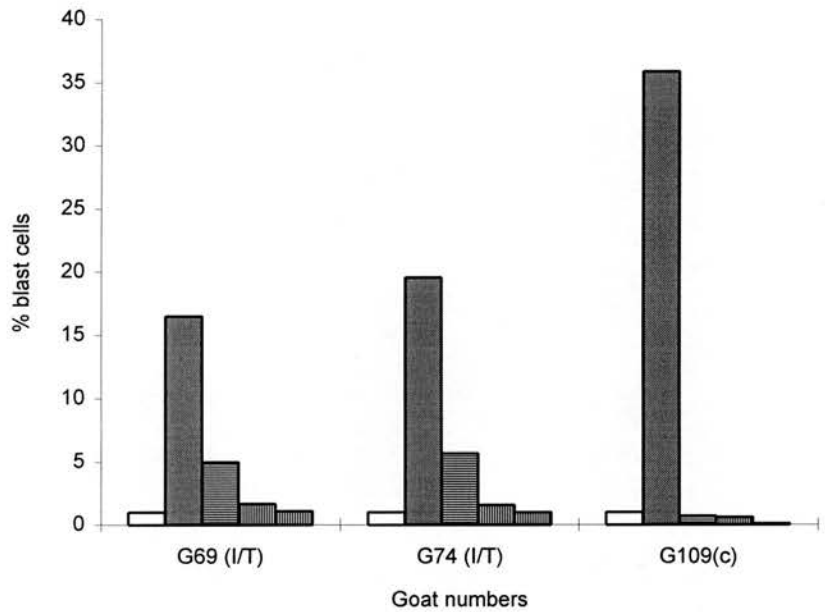



Figure 6.4. Proliferatve responses of PBMC from I /T goats to Cowdria EBs, recombinant antigen (35kDa GroEL fragment) and Concanavalin A.
Goats G 69 & G74 were immunisedby infection treatment and G109 was a naive control. Responses to 5 μg/ml Concanavalin A ( ), 15 μg/ml of,homologous EBs ( ), recombinant (35kDa) : 5 μg/ml ( ), and 2.5 μg/ml ( ) and un-stimulated controls ( ).

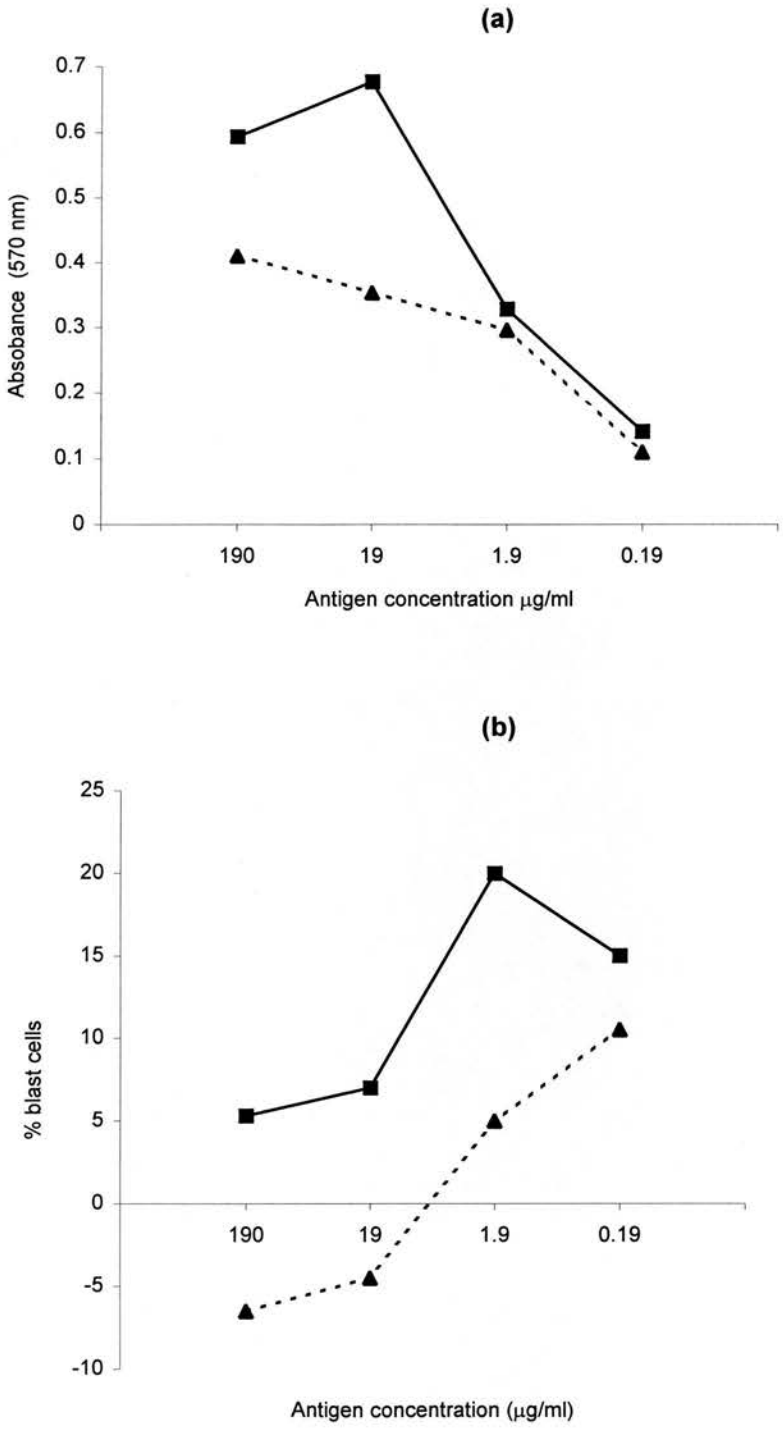



Figure 6 .5. Comparison of the effect of live or inactivated homologous EBs (Welgevonden) on the proliferative response of PBMC from one I/T goat (G74) . Responses measured by MTS assay (a) or by counting lymphoblasts using cytospin smears (b).PBMC stimulated with live EBs (▲) or inactivated EBs ( ■ ).

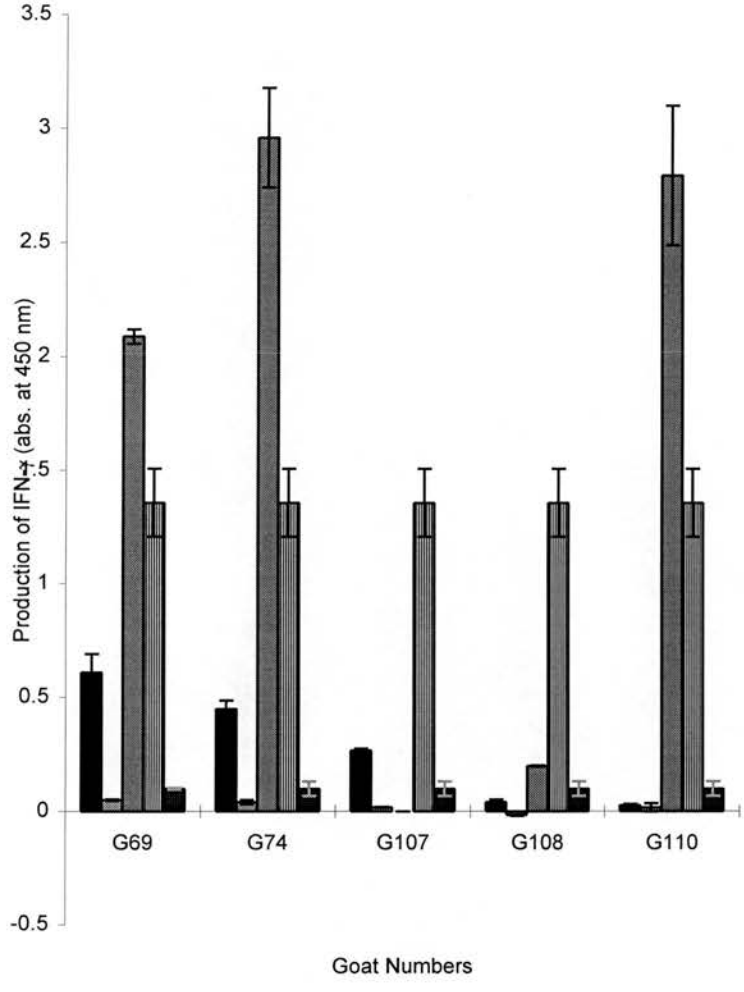



Figure 6.6. Production of IFN-γ *in vitro* by PBMC from goats immunised with live EBs (G69 & G74),or with inactivated EBs (G107 & G108) stimulated with 15 μg/ml of homologous EBs ( ■ ) . or or 5 μg/ml of recombinant (35kDa) antigen ( ▒ ), control positive ( ▒ ) Concanavalin A ( ▒ ), and negative control ( ▒ ).

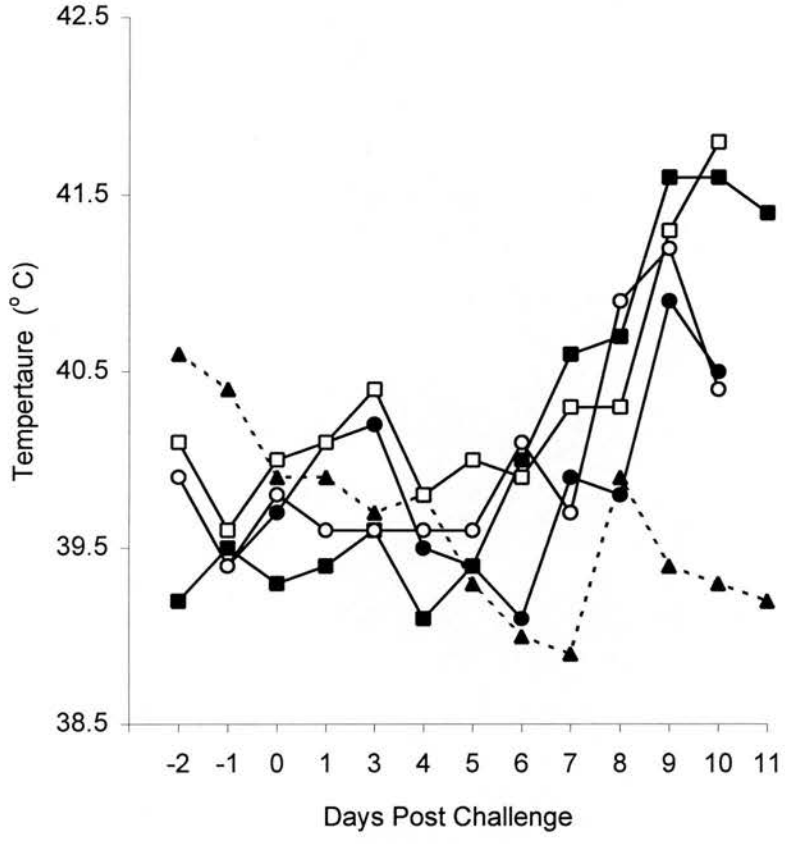



Figure 6.7. Daily rectal temperatures (°C) of goats before and after challenge with virulent of *C. ruminantium* (Welgevonden).
Temperature reactions of I/T goat G74 (▲) , IEB goat G107 (■ ), IEB goat G108 ( ● ), control goats G109 ( □ ), and control goat (○ ).

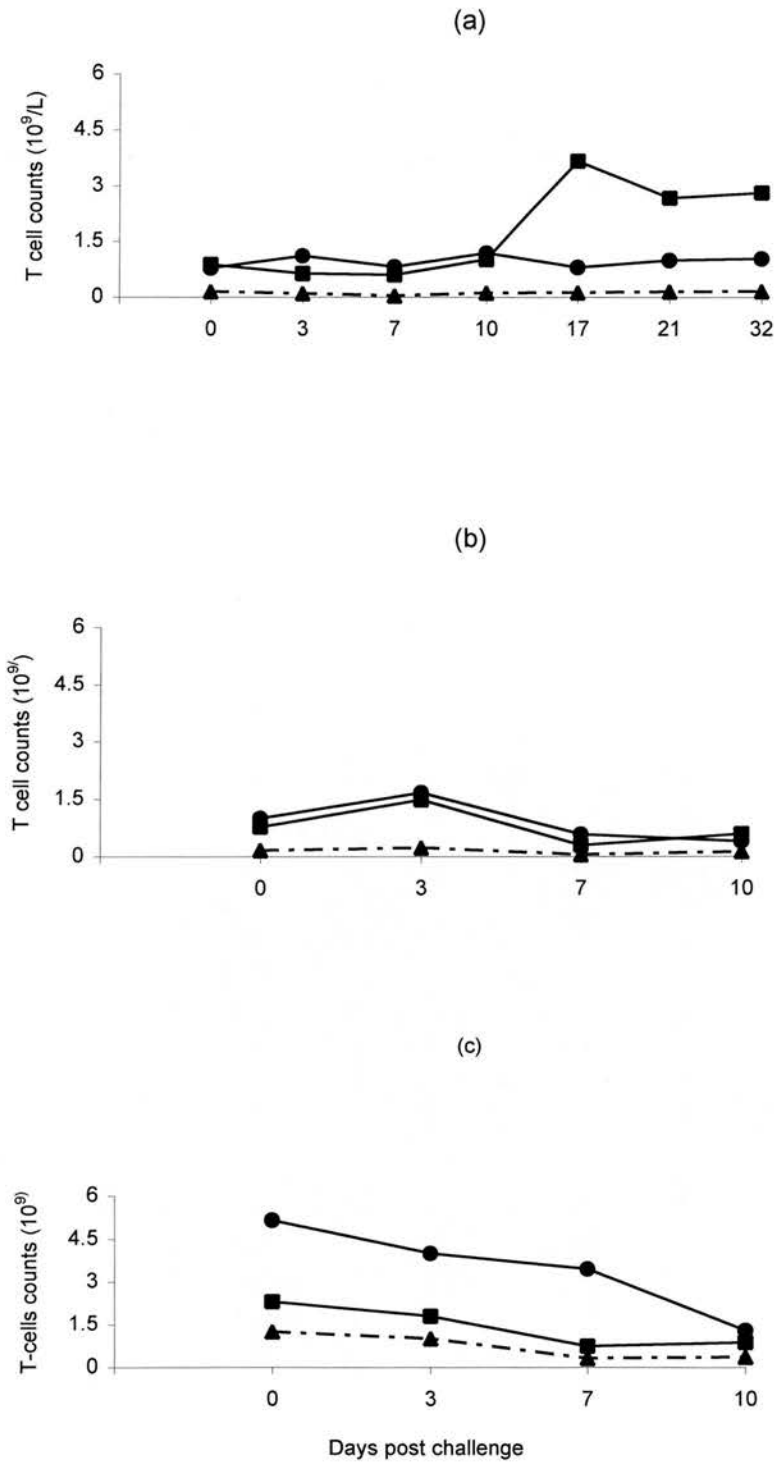



Figure 6.8 a to c. Effects of challenge on peripheral blood T-cell populations of three immunised goats. Counts of I/T goat G74 (Figure 6.9a), of IEBs goat G107 (Figure 6.9b) and of IEB goat G108 (Figure 6.9c).
$CD4^+$ counts ( ● ), $CD8^+$ counts ( ■ ) and γδ counts ( ▲ ).

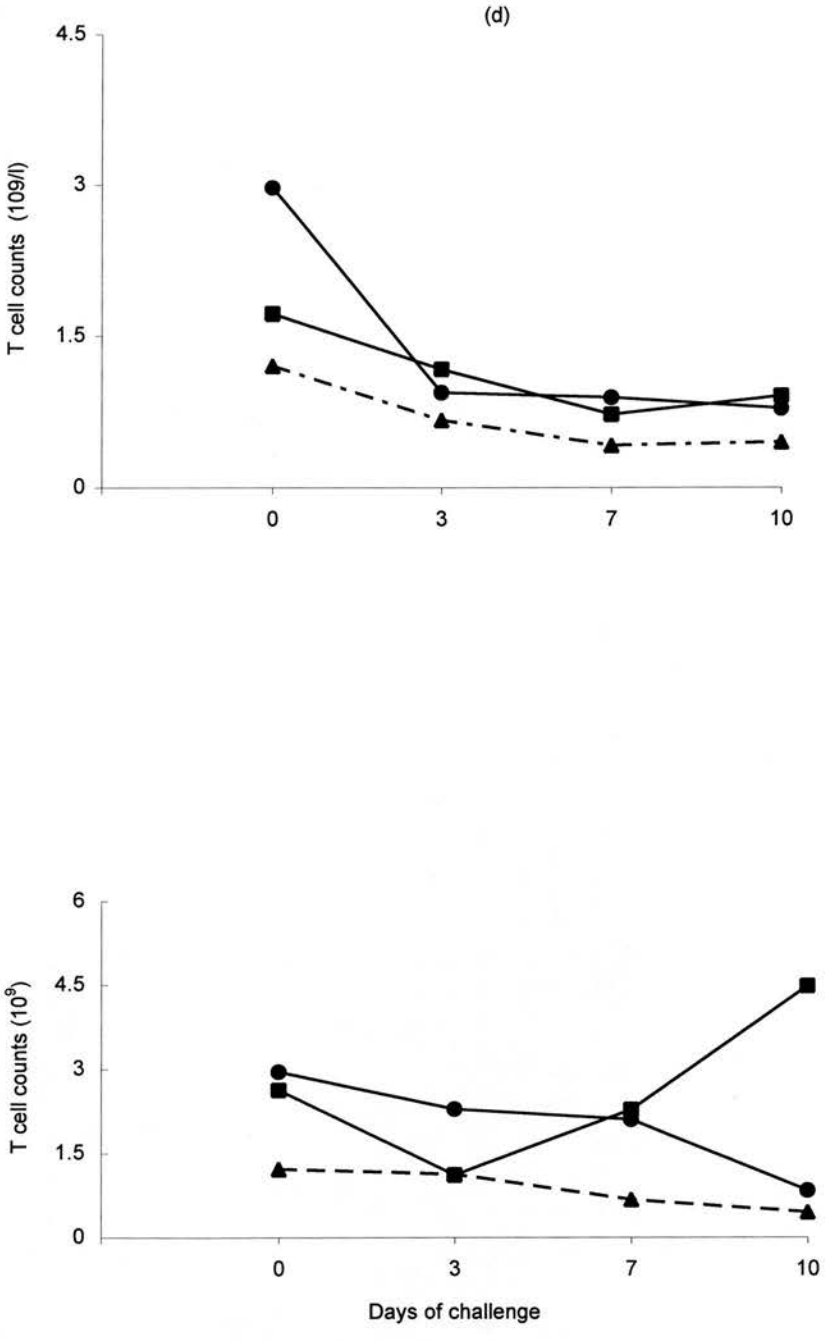



Figure 6.8d and e. Effects of challenge on peripheral blood T cell populations of the control goats. Counts of control goat G109 (Figure 6.9d), and control goat G110 (Figure 6.9e).

CD4 counts ( ●), CD8 counts ( ■ ) and g/d T cells (▲ ).

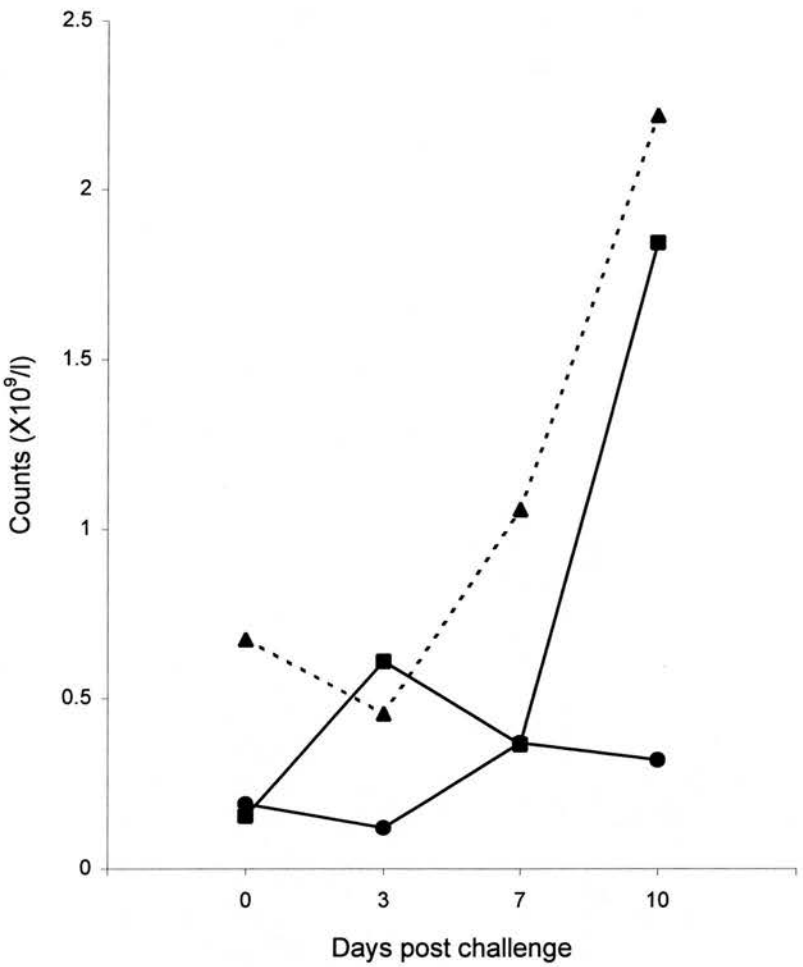



Figure 6.9a. Effect of challenge on the monocyte counts in peripheral blood of immunised and control goats.
Counts of the I/T goat ( ● ), mean counts of the IEB group ( ■ ) and mean counts of the control group ( ▲ ).

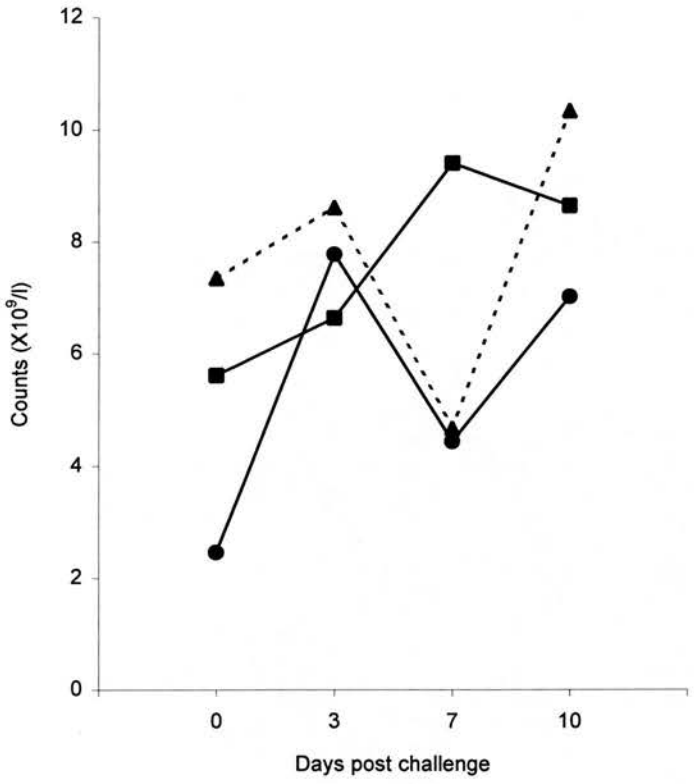



Figure 6.9b. The effect of challenge on the neutrophil counts in peripheral blood of immunised and naivecontrol goats.
Counts of the I/T goat ( ● ), mean neutrophil counts of the IEB group ( ■ ) and mean neutrophil counts of the control group ( ▲ ).

# CHAPTER SEVEN.

# IMMUNISATION OF MICE AND SHEEP WITH RECOMBINANT 58KDA PROTEINS (GroEL)) OF *COWDRIA RUMINANTIUM.*

## *7.1 Introduction*

Protective immunity against heartwater develops after animals recover from natural and experimental infections (Stewart, 1987). It is also achieved by intravenous immunisation of ruminants with predetermined doses of live *C. ruminantium,* administered as blood stabilates or ground-up tick supernatants (Oberem and Bezuidenhout, 1987a; Camus *et al.*, 1996). Inoculated animals are allowed to develop fever of above 40$^{o}$C and treated on the second day of fever with one or two doses of oxytetracycline at 20 mg/kilogram body weight (Jongejan *et al.*, 1993b). Recovered animals are solidly immune to homologous challenge for a period ranging from 4 months to 3.5 years and are partially resistant to heterologous challenge (Camus and Barre 1988; Jongejan *et al.*, 1993c; Uilenberg 1983). The live vaccine is prepared from the Ball 3 stock (Haig 1952; Jongejan and Thielemans, 1989b). Live vaccines however, are limited by a number of factors: They are expensive to produce and maintain, they require technical personnel to administer, other tick-borne diseases such as East coast fever, babesiosis and anaplasmosis can be transmitted and they can cause direct losses from cowdriosis (Oberem and Bezuidenhout, 1987a; Camus *et al.*, 1996). Another limitation on their usefulness is the low level of cross-protection against several South African strains following

vaccination with Ball 3 stock (Du Plessis *et al.*, 1989b; Du Plessis *et al.*, 1990a, Jongejan and Thielemans, 1989b).

Two other vaccine preparations which have been used to immunise ruminants against heartwater are, inactivated elementary bodies (Martinez *et al.*, 1993, 1994; Mahan *et al.*, 1994a; 1995; Tafesse, 1992) and attenuated organisms (Jongejan, 1991a; Jongejan, *et al.,* 1993b; Gueye *et al.,* 1994). Animals receiving attenuated organisms are solidly protected against virulent homologous challenge (Jongejan 1991, Jongejan *et al.* 1993b). On the other hand, not all animals receiving inactivated organisms are protected against virulent homologous challenge (Mahan *et al.*, 1995b, Martinez *et al.*, 1994; Tafesse, 1992). The latter immunogens are limited by the high cost of production, and low level of cross-protection among *Cowdria* isolates. Additionally, for the attenuated culture vaccines only the Gardel and Senegal stocks have been attenuated(Jongejan 1991; Jongejan *et al.,* 1993c; Gueye *et al.*, 1994). The Welgevonden stock which confers a broader level of cross-protection by infection/treatment has not been attenuated (Du Plessis *et al.*, 1989b, Camus *et al.*, 1996). However, attenuation may not necessarily preserve immunogenicity. In recent studies in Kenya (Ngumi, 1997), low virulence *Cowdria* were not protective against high virulence isolates but the converse was the case.

The most desirable approach to control heartwater is the development of a 'broad' spectrum vaccine composed of a number of protective antigens with conserved epitopes present in all the strains of *Cowdria ruminantium.* Animals which recover from natural or experimental heaartwater infection develop antibodies

which detect several immunogenic proteins with relative molecular masses of 14kDa to 118kDa (Barre and Camus, 1987; Semu *et al.*, 1992, Section 4.3.1). A number of these antigens, such as the 32kDa protein (MAP1), (Jongejan and Thielemans, 1989a), the 21kDa protein (MAP2) (Mahan *et al.*, 1994b) and the 58kDa protein (Lally *et al.*, 1995) are immunodominant and conserved. The three antigens are found on the surface of the EB as determined by biotin labelling (Section 4.3.1). The 58kDa protein is a heat shock protein (Hsp) which belongs to a family of heat shock proteins, also considered as molecular chaperonins, designated Hsp 60 (Cpn 60) which have homologues in all other bacteria with published sequences, for example *Rickettsia tsutsugamuchi* and *E. coli* (Stover, Marana and Oaks, 1990). To date, relatively little is known about the importance of some of the immunogenic proteins of *C. ruminantium* with respect to protective immunity in susceptible ruminants. Purified native, recombinant and synthetic peptide antigens are useful in determining the role played by specific antigens of *C. ruminantium* in immune responses of ruminants. Native antigens are obtained by growing large quantities of the organism in tissue culture followed by separation with affinity, ion exchange or size exclusion chromatography. Production of large amounts of *C. ruminantium* is very expensive and time consuming. Genes for three immunodominant antigens of *C. ruminantium* have been cloned and expressed, they are: the 32kDa protein (MAP1) van Vliet *et al.* (1994), the 21kDa (MAP2) protein (Mahan *et al.*, 1994b) and the 58kDa heat shock protein (GroEL) (Lally *et al.*, 1995). The GroEL antigen is recognised by antibodies in sera of animals which have recovered from live infection and in from those

immunised with inactivated elementary bodies. The gene encoding the 58kDa antigen of *C. ruminantium* was cloned and expressed in *E. coli* (JM109) as a recombinant protein encoded by plasmid pBluescript Cr9.4 (Lally *et al.*, 1995). The successful cloning and expression of the gene coding for the 58kDa Hsp permitted investigation of the antibody and cellular responses of mice and sheep immunised with this antigen. Studies of protection against heartwater by immunisation with recombinant *Cowdria* antigens have not been published, this study represents an investigation of the immunogenicity and protectve capacity of a recombinant heat shock protein in mice and sheep against heartwater. The objectives of this studies were

1. To determine if immunisation with recombinant 58kDa or antigen fragments of this protein protected mice and ruminants against virulent challenge.
2. To determine if immunisation led to a reduction in level of rickettsial infection in endothelial cells of infected sheep.
3. To determine the nature of cellular and humoral responses to GroEL induced by immunisation.

## 7.2. Materials and Methods

### *7.2.1. Experiments with recombinant 58kDa Hsp antigen of C. ruminantium*

#### *7.2.1.2. Experiment 1. Immunisation of mice with bacterial lysates containing recombinant 58kDa Hsp (JM109/pBSCr9.4) and plasmid control (JM109/pBS).*

The aim of this experiment was to immunise mice to achieve a detectable antibody response to recombinant 58kDa Hsp and challenge them to test for

protection. *E. coli* JM109 strain carrying the plasmid (pBSCr9.4) encoding the 58kDa antigen and control bacteria carrying the Bluescript plasmid (JM109/Bluescript) were cultivated and the presence/absence of the recombinant ascertained (Section 3.6). Then the bacterial antigens were prepared as described earlier (Section 3.13) and used to immunise the mice. Six groups of mice were inoculated as shown in Table 7.1.

Primary and booster inoculations were administered subcutaneously. Boosters were given on days 14 and 21 post inoculation (PI).

#### *7.2.1.3. Experiment 2. Immunisation of mice with bacteria hosting the recombinant 58kDa Hsp antigen.*

The aim of this experiment was to determine if the recombinant 58kDa Hsp expressed by bacteria in the native form could confer greater protection than the bacterial lysate which was prepared under denaturing conditions. In this experiment mice were inoculated with *E. coli* cultures expressing the recombinant 58kDa Hsp JM109/pBSCr9.4 or control bacterial cultures containing the plasmid (JM109/pBS). Two groups (G and H) of 10 mice each were used. Mice in group G were inoculated 4 times with 0.1 ml LB broth culture of *E.coli* (JM109/pBSCr9.4) containing $5.2x10^5$, $1.8x10^5$, $3x10^5$ and $1.2x10^5$ bacteria per dose administered subcutaneously on days 0, 3, 7, and 14 PI respectively. Finally mice in this group were inoculated with $1.0x10^5$ organisms intramuscularly (IM) in the quadriceps on day 21 PI. Mice in the control group (group H) were inoculated 4 times with $7x10^5$ of bacteria

(JM109/pBluescript) containing the plasmid (pBScript) in 0.1 ml LB broth subcutaneously.

Blood samples for serum preparation from all groups of mice were collected on days 0, 14, 21, 28, and 34 PI as described in section 3.3. Sera were examined for the presence of antibodies by Western blotting as described earlier (Section 5.2.1.4) and ELISA (Sections 5.2.3) except that the conjugate used was a rat anti-mouse IgG Fc specific HRP (Sigma).

#### *7.2.1.4. Experiment 3. Immunisation of mice with 35kDa fusion protein.*

The aim of this experiment was to investigate the level of protection induced by immunisation with the 35kDa GroEL. Another reason was that large quantities of this purified protein were available for immunisation whereas the 58kDa full length protein could not to be expressed for purification.

Seven groups of 5 mice each (Groups A to G) were inoculated with the 35kDa subclone antigen or with adjuvants only as shown on Table 7.2. The antigens were prepared as described in Section 3.14. Mice in group A were each inoculated 3 times with 50 μg protein of the purified 35kDa subclone protein. Antigen used for primary immunisation of this group were prepared in FCA and boosters were prepared in FIA while, those in group B were inoculated with the same amount of purified 35kDa prepared in an oil adjuvant (Mantonide-ISA50) which had been previously used successfully to immunise animals against heartwater (Martinez, *et al.*, 1993). All the inoculations were administered subcutaneously in 0.2 ml amounts per mouse (Table 7.2).

Mice in groups C and D were inoculated 3 times with 250 μg/ml of bacterial lysates containing pTrcHis0.7 or the plasmid control respectively. The bacterial lysates were prepared as described in Section 3.6 then they were prepared for mouse inoculation in either FCA or Mantonide ISA50 as described for the purified recombinant 35kDa (subclone) antigen.

Blood samples for serum preparation were collected on as described for previous groups (Section 7.2.1.3) and tested for seroconversion by Western blotting and indirect ELISA as described in Section 4.2.5 and Section 5.2.1.4 respectively using goat anti-mouse conjugate $IgG_1$ horse radish peroxidase (whole molecule)

### 7.2.1.5. *Challenge and monitoring of mice after challenge.*

Mice in these 3 experiments were each challenged with $1.2 \times 10^3$ live virulent EBs in 0.1 ml culture of *C. ruminantium* (Wlgevonden stock) administered intravenously in the tail vein 35 days after immunisation. Survivors of primary challenge were challenged again with $4 \times 10^3$ live virulent EBs 30 days later.

After challenge mice were observed for clinical signs of heartwater. Post mortem examination was carried out on mice which did not survive challenge, presence of pulmonary oedema, pleural effusions, ascites and hepatic changes were recorded. Impression smears prepared from lungs, brain and liver were fixed in methanol for 5 minutes, stained with 5% Giemsa (Modified Wrights stain) and examined for *C. ruminantium* by light microscopy ( Leitz LaborLux-K microscope) at a magnification of 400x.

### *7.2.2. Experiment 4. Inoculation of sheep with bacterial lysates containing recombinant 58kDa Hsp (JM109/pBSCr9.4/GroEL) antigen of C. ruminantium.*

The aim of this experiment was to investigate the cellular and antibody responses of sheep immunised with 58kDa Hsp recombinant protein (GroEL) of *C. ruminantium* and to determine if they showed evidence of protection against virulent homologous challenge.

#### *7.2.2.1. Experimental animals.*

Twelve Merino/Scottish Blackface cross rams (Section 3.1.3) were used in this experiment. Six rams were selected randomly (3 from each age group) and placed in two groups. Sheep in the first group were immunised with bacterial lysate containing the recombinant 58kDa Hsp (JM109/pBSCr9.4). The immunised group was made up of sheep numbers S66, S69, S71, S72, S74 and S76. Sheep in the second group served as controls and were inoculated with bacterial lysate containing the plasmid control (JM109/pBS). The controls were sheep numbers S67, S68, S70, S73, S75 and S77.

*7.2.2.2.* ***Inoculation of antigens***. Bacterial lysates containing the plasmid (pBS) with or without the 58kDa Hsp were prepared as described earlier (Section 3.6). Then they were mixed with equal volumes of FCA or FIA and prepared as antigen/adjuvant suspensions as described in Section 3.14.

Sheep in the first group were immunised with 250 μg of recombinant 58kDa Hsp (pBSCr9.4) prepared in FCA. Each sheep in the control group was inoculated

with 250 μg protein of bacterial lysate containing the plasmid control (pBS) prepared in FCA. Primary inoculations on day 0 were administered intramuscularly in the quadriceps. After primary inoculations, sheep in each respective group received 5 booster inoculations at days 14, 21, 34, 64 and day 121 PI administered subcutaneously in the neck region.

#### *7.2.2.3. Sample collection from immunised sheep.*

Serum samples were collected from all sheep as described earlier (Section 3.3) at days 0, 14, 21, 28, 34, 64, 82 and day 143 PI. Seroconversion was monitored by indirect ELISA and Western blotting. Blood samples for cell proliferation assays and flow cytometric analysis were collected on days 21 and day 64 PI.

#### *7.2.2.4. Test for specific antibody production to 58kDa Hsp in sera of immunised sheep by ELISA and Western blotting.*

Detection of specific antibody responses of immunised sheep by indirect ELISA was carried out as described earlier (5.2.5) with the following modifications: The antigen used was a purified 35kDa recombinant subclone protein of the recombinant 58kDa Hsp (GroEL). It was purified as described earlier (Section 3.7).

Sera dilutions of 1:200 were used, a peroxide conjugated donkey anti-sheep IgG whole molecule (Sigma) was used to detect antibody binding. Reagents, and procedures were the as described eaerlier (Section 5.2.5). The mean OD values of 2 tests were calculated and the means were used to analyse the serological responses to the recombinant antigen.

Sera from immunised sheep were also examined for $IgG_1$ and $IgG_2$ responses using an indirect ELISA as described in Section 5.2.3.

Western blotting was used to verify that the specific antibodies in sera, of immunised sheep detected by ELISA recognise EB antigens of *C.ruminantium*. The test was carried as described earlier (Section 4.2.5) except that the conjugate used was donkey anti-sheep IgG whole molecule horse radish peroxidase (Sigma).

### *7.2.2.5. Cellular immune responses to immunisation with 58kDa recombinant antigen.*

***Proliferative responses.***

Proliferative responses of PBMC obtained at days 21 and 64 PI from 5 randomly selected sheep from the immunised group and the two control sheep were carried out *in vitro*. Briefly: Fresh PBMC were purified as described in Section 3.2. Then stock cultures seeded with 1x $10^6$ cells/ml in complete RPMI 1640 medium were stimulated with 1, 2.5, 5, 10, and 15 μg/ml of recombinant 58kDa Hsp or bacterial lysates containing the plasmid only (Section 3.6). The cultures were incubated in triplicate in aliquots of 200 μl/well in 96 microtitre well plates (Nunc, Denmark) as described in Section 6.2.3. After 72 hours, 100 μl supernatants from each culture were collected and assayed for interferon gamma (IFN-γ) as described earlier (Section 6.2.6). Additionally three more PBMC cultures from the sub-groups used above were stimulated with 5 μg/ml of purified 35kDa subclone antigen (Section 3.7), 15 μg/ml EB protein, or 5 μg/ml Concanavalin A supernatants colected on day tested for IFN-γ. After five days of incubation cultres were tested for

proliferation by MTS assay as described in Section 6.2.4. The results were expressed as the median OD values for three tests.

***Flow cytometric analysis of antigen stimulated T cells.***

Following *in vitro* stimulation with 58kDa protein, the surface phenotype of PBMC from a randomly selected subgroup of 4 immunised sheep and two controls were analysed by indirect fluorescence staining using specific MAbs ST4, SBU, and 86D (Table 6.1) which recognise ovine $CD4^+$, $CD8^+$ and γδ T cell receptors respectively. Three stock cultures of PBMC from each sheep were stimulated with 15 μg/ml of recombinant 58kDa Hsp protein or control plasmid (pBS/JM109) or *Cowdria* EBs respectively. After 5 days of incubation PBMC were washed and subdivided into three samples. Then one each was stained for $CD4^+$, $CD8^+$ and γ/δ T cells and analysed on a fluorescence-activated cell scanner (FACS) as described in Section 6.2.6.

***Assays for cytotoxic activity after immunisation with 58kDa recombinant.***

The aim of this experiment was to determine if Cytolytic activity (CTL) could be detected in immunised animals before and after challenge providing that the animals recovered, and to compare responses after challenge.

Non-radioactive cytotoxicity assays were performed with lymphocytes as effectors against autologous endothelial cells as targets. They were obtained from three sheep (S66, S69 and S72) in the immunised group. PBMC from these sheep were collected 5 days after booster inoculations on day 69 and 87 PI, purified as

described earlier (Section 3.2) and depleted of adherent cells to obtain effectors as described in Section 3.2.1.

The effectors were divided into two equal fractions; one was used without antigen stimulation and the other was stimulated with 15 μg/ml EB for 5 days before use.

The cytotoxic assays were carried out using a lactate dehydrogenase (LDH) assay (The Cytotox 96® Non-radioactive Assay, Promega, Madison, WI). Lactate dehyrogenase release was measured in an enzymatic assay following the manufacturers instructions with some modifications. The target cells were prepared as described in Section 3.16.

Optimisation of the targets was carried out according to the manufacturer's instructions (Promega, Madison, WI, USA) with some modfications to suit the assay. The endothelial cell cultures were maintained in GMEM supplemented with 20% foetal calf serum (Sigma). The optimum number of targets (endothelial cells) used were $1.5 \times 10^4$ cells/well. Infected targets were used to test for cytolytic activity while un-infected targets served as controls. The tests were carried out using a range of effector:target ratios of 20:1 to 0.312:1 aliqouts of 200 μl seeded in 96 well microtitre plates. The mean OD of 4 tests taken at 492nm were used to determine if lysis had occurred and specific lysis was calculated as in standard $^{51}Cr$ release as described by Brander *et al.*, (1993) and was:-

100 X (experimental LDH release subract spontaneous LDH release)/(maximum LDH release subract spontaneous LDH release). Results were expressed as percent lysis of targets.

### *7.2.2.6. Challenge of sheep with live virulent Cowdria EB suspension.*

Sheep were placed in isolation and challenged on day 143 PI (34 days after the last booster). Then each sheep was inoculated intravenously in the jugular vein with 2 ml of a virulent homologous culture of *C. ruminantium* (Welgevonden stock) containing 3.0 x $10^5$ EBs. Prior to challenge, blood samples were taken from each sheep for haematology, and flow cytometric analysis. Samples were taken in EDTA or heparinised vacutainer tubes. After challenge further sampling was carried out at days 3, 7 and 12 PC and monitored for changes in white blood cell counts.

### *7.2.2.7. Peripheral blood cell counts after challenge.*

Total WBC and differential counts were carried out at the Haematology Laboratory, Department of Veterinary Clinical studies, Royal (Dick) School of Veterinary Studies, University Edinburgh as described in Section 6.2.7.

Mononuclear cell counts for all the sheep were determined after challenge following the same procedures described earlier (Section 6.2.8.1). Additionally the surface phenotypes of T cells in peripheral blood obtained from the immunised and control sheep at days 0, 3, 7 and 12 PC were determined (Section 6.2.7). The percentage of $CD4^+$, $CD8^+$ and γδ T cells were transformed into absolute numbers using the total and differential counts of lymphocytes in peripheral blood (section 6.2.8.1).

After challenge, lymphocytes (effectors) were obtained from two immunised sheep (S66 and S69) at day 3 and 7 PC and used to carry out cytotoxic assays as described in Section 7.2.3.5.

### *7.2.3. Statistical analysis.*

The medians of the daily counts of the T cells, monocytes, and neutrophils were used to analyse day to day, sheep to sheep and inter-group differences using Fischer's exact test, student t-test or Anova two way analysis of the variance.

## 7.3. Results

### *7.3.1. Evidence that bacterial lysates contained the 58kDa Hsp recombinant antigen*

Western blot analysis of bacterial lysates containing the 58kDa recombinant protein revealed that the bacterial lysates contained an antigen of 58kDa that reacted with immune sera obtained from an I/T goat. No reaction was observed with bacterial lysates of the control pBS plasmid (Figure 7.1).

### *7.3.2. Development of antibodies to 58kDa Hsp after immunisation of mice with 58kDa Hsp recombinant antigen.*

Sera collected at days 0, 14, and 21 PI from mice immunised with bacterial lysates containing recombinant 58kDa Hsp did not show evidence of antibodies to the 58kDa antigen. Sera collected from day 28 PI onwards reacted with the homologous 58kDa Hsp protein of the *Cowdria* EB (Figure 7.2 lanes 6 and 7). Sera collected from mice in the control group did not react with this antigen (Figure 7.2 lanes 10 to 14)

### *7.3.3. Effects of challenging mice immunised with bacterial lysates containing 58kDa Hsp and controls inoculated with plasmid (pBS).*

A total of 4 out of 29 mice immunised with recombinant 58kDa Hsp as lysate (Group A, B) or bacteria (Group G) survived virulent homologous challenge (Table 7.3). Two of the surviving mice had been immunised with bacterial lysates of the recombinant 58kDa prepared in FCA or FIA and the others had been immunised with bacteria (JM109/pBCr9.4) expressing this recombinant antigen. None of the controls

(Group C, D, E, F and F) survived challenge with virulent homologous stock (Table 7.3).

The incubation period of heartwater in the immunised and control groups ranged between 8 and 12 days. All mice showed classical clinical signs of *Cowdria* infection before death. Death occurred 24 hours after the appearance of the clinical signs. The survivors were depressed and had raised hair for 3 days before recovery. No respiratory signs were noticed in mice which survived whereas all non survivors had respiratory signs.

Western blotting with sera from 4 mice which survived primary challenge gave very strong reaction with the native 58kDa Hsp of the EB of *C. ruminantium* in contrast to sera after recombinant immunisation and other antigens of the EB (Figure 7.3, lanes: 6 and 7) indicating an active response to *Cowdria*. The presence of reactions to at least five EB antigens not recognised by pre-challenge sera indicate that the animals made an active immune response to challenge. The four mice which survived the first challenge also survived a second challenge using $4 \times 10^3$ EBs.

Analysis for differences between groups in the survival time/day of death showed that immunised and the control groups to survive showed that immunised mice died earlier than the controls (P =0.015) (Table 7.4).

### *7.3.4. Evidence of purification of the recombinant 35kDa protein (subclone of GroEL) of C. ruminantium.*

SDS-PAGE analysis and Coomassie blue staining of gels containing eluates obtained after purification of the recombinant 35kDa protein revealed that the second and third eluates were free from bacterial protein contamination (Figure 7.4a lanes: 5

and 6). Western blots containing purified 35kDa antigen, plasmid controls or EBs screened with sera from an immune goat showed that lanes with purified antigen and the EB antigens reacted with specific antibodies from recovered animals to respective antigens (Figure 7:4b lanes: 2 and 3).

### *7.3.5. Effect of challenge with virulent culture of C. ruminantium (Welgevonden stock) on survival of immunised mice.*

A total of 7 of 20 mice inoculated with the 35kDa subclone (Histag) protein survived the first challenge with a virulent culture of homologous stock (Table 7.5). Three of the survivors had previously been primed with the purified recombinant antigen, and 4 had received bacterial lysate incorporated in FCA. Four of five FCA controls also survived this challenge (Table 7.5). To ensure survival was not the result of mal-administration of the challenge, a second challenge was given and one of the FCA inoculated survivors and none of those immunised with 35 kDa antigen survived. The incubation period for all the groups was 9.8±1.2 days.

The post mortem findings of mice after challenge were similar to those seen in classical heartwater. Examination of lung, liver and brain impression smears collected at necropsy and stained with Giemsa showed the presence of a low number of *C. ruminantium* colonies in lung macrophages and rarely in the brain and liver.

A summary of the results of the mouse immunisation experiments is given in Table 7.6. A total of 4 out of 49 mice inoculated with full length or subclone GroEL antigen survived challenge with virulent homologous stock of *C. ruminantium*. This gives a protection rate of 27.5% against primary and secondary challenge.

### *7.3.6. Results of sheep immunisation experiment*

#### *7.3.6.1. Serological responses of sheep inoculated with the recombinant 58kDa Hsp.*

***(a) Antibodies which developed following immunisation of sheep with recombinant 58kDa Hsp protein recognise epitopes on the 35kDa subclone.***

The mean ELISA OD values of sera from sheep immunised with recombinant 58kDa Hsp increased progressively from day 0 to the highest values on day 34 PI (Figure 7.5). Individual variations were however observed between individual animals with OD values ranging from 0.1 to 0.4 within this group (Appendix C: Figure 1 ). The ELISA OD values of sera from the controls were lower than 0.1 except for two sheep (S68 and S70) which had OD values higher than 0.15 (Figure 7.5).

***(b) Development of antibodies to 58kDa Hsp after immunisation of sheep with bacterial lysates containing recombinant 58kDa Hsp protein.***

Inoculation of sheep with bacterial lysates containing the 58kDa Hsp recombinant antigen led to the development of specific antibodies which reacted strongly with the homologue in the EB of *C. ruminantium* (Figure 7.6a) by Western blotting. Antibodies to this antigen were detectable in sera collected from day 14 post inoculation onwards. Western blots probed with sera from sheep inoculated with control plasmid did not show evidence of anti-*Cowdria* 58kDa antibodies (Figure 7.6b, lanes: 2 to 7).

### *7.3.6.2. Proliferative responses of PBMC from immunised and control sheep PI. to bacterial lysates containing recombinant 58kDa Hsp protein of C. ruminantium*

PBMC from both immunised and control sheep responded in a dose dependent manner to bacterial lysates containing the recombinant 58kDa Hsp (pBSCr9.4) but not with that carrying the plasmid control antigen alone (Figures 7.7a, b, c). The highest OD values were obtained with 15 μg/ml of pBSCr9.4 as antigen.

### *7.3.6.3. Evidence of cytotoxic activity after immunisation.*

There was no evidence of lysis of targets (Ec) by un-stimulated or stimulated effectors. The percent lysis of Ec by different effector:target ratios were lower 0%. This was caused by higher LDH spontaneous release OD values than those of the experimental LDH release.

### *7.3.6.4. Challenge.*

#### *Clinical signs.*

Classical signs of heartwater were observed in all cases of immunised and control sheep after challenge. Three sheep in the immunised group developed clinical signs on the fifth day post challenge (PC). Fever was recorded first in 2 of 6 sheep in the immunised group on day 6 PC and by day 7 PC all sheep in this group had developed fever of 40.7°C and higher (Table 7.7). In the control group, 1 of 6 sheep developed fever on day 6 PC, 4 of 6 on day 7 and 1 of 6 on day 9 PC respectively. Ten sheep in both groups developed fever by day 7 PC (temperatures ranging from 40.8°C to 41.7°C). The highest temperature (42.5°C) reaction recorded in both groups was in a control sheep. The maximum rectal temperature for the

control group was 42.2 ± 0.6°C (mean ± SD) and that of the immunised group was 42.3 ± 0.8°C. The duration of fever in the immunised group was 6 ± 3 (mean ± SD) days and 6 ± 1 days for the control.

The mean temperature for the immunised group remained higher than that of the control group from day 5 PC except on days 0, 3 and 11 PC (Figure 7.8).

The mean incubation period for the immunised group was 7 ± 1SD days and that of the control was 7.5 ± 1.5SD days. The immunised group developed fever earlier than the control group and this fever remained until death.

Two sheep in the immunised group (S66 and S69) developed nervous signs including aggression, knocking of head on objects, turning in circles, grinding of teeth, nystagmus and mild convulsive seizures and were euthanised by administration of Pentobarbitone intravenously. One control sheep (S77) developed a foul smelling diarrhoea two days before it died. None of the immunised animals survived the challenge (Table 7.7a and b).

Sheep in the immunised and control groups died on different days after development of fever (Table 7.7a and b). In general fever lasted 3 days longer in the immunised group than controls.

### *7.3.6.5. Effect of challenge on peripheral blood leukocytes counts.*

#### *CD4$^{+}$ populations after challenge.*

The mean $CD4^{+}$ counts of the immunised group increased above pre-challenge counts (day 0 counts) to the highest counts at day after challenge 3 (Figure 7.9) followed by a highly significant ($F=9.11$, $P<0.05$, Table 7.8) reduction at day 7

PC. The counts dropped further on day 12 PC (Figure 7.9). Highly significant differences between sheep in the immunised group ($F=9.11$, $P<0.05$) were also observed in this group. The mean $CD4^+$ counts of the control group increased on day 3 PC above day 0 counts, followed by a highly significant ($F=16.79$, $p<0.05$) reduction on day 7PC (Table 7.8) then, the counts restored to the high level noted on day 3 at day 12 PC. In contrast to the immunised group there were no significant differences between counts of sheep in the control group ($F=2.66$, $P>0.005$). The $CD4^+$ counts of individual animals in the immunised and control groups were characterised by individual variations (Appendix B Table 8a, b)

Analysis for group to group differences between the median $CD4^+$ counts of the immunised verses the control groups revealed no significant differences ($t=0.99$, $p>0.05$).

***$CD8^+$ counts after challenge.***

The mean $CD8^+$ T cell counts of the immunised group increased from day 0 counts to about twice on day 3 PC, followed by a reduction in numbers to counts lower than that of day 0 at day 7 PC (Figure 7.10). Unlike the reduction noted in median counts of $CD4^+$ in this group the reduction in $CD8^+$ counts was not significant ($F=2.81$, $p>0.05$). There was another increase to about 2.5 times day 3 counts at day 12 PC (Figure 7.10). It was also noted that differences between median counts of sheep in the immunised group were not significant ($F=3.09$, $p>0.005$). The mean $CD8^+$ T cell counts for the control group behaved in a similar manner (Figure 7.10). In this group no significant differences between median counts of day 3 vs day 7 ($F=2.38$, $p>0.05$)

or median counts sheep to sheep(F=1.95, p=>0.05), (Table 7.9). Individual variation were noted in $CD8^+$ counts of animals in the immunised and control groups (Appendix B Table 10a, b ).

Analysis for group to group differences between the median $CD8^+$ T-cell counts of the two groups revealed no significant differences (t =-0.9, p>0. .05).

Determination of the $CD4^+$:$CD8^+$ ratios of the immunised group PC showed that on day 3 PC the median ratio was 4:1, on day 3PC, 6:1 on day 7 PC and 3:1 on day 12 PC (Appendix B Table 12). In the control group the ratios were 3:1 on days 3 and 7 PC and 2:1 on day 12 PC (Appendix B. Table.12).

***γδ T cell counts after challenge.***

The mean γδ T cell counts in peripheral blood of sheep in the immunised and control groups decreased from day 3 counts to he lowest on day 10 PC. Pre-challenge counts in all the groups were not available. Analysis for differences between median counts of the two groups revealed no significant differences. (F=1.869, p>0.05).

***Monocyte and neutrophil counts after challenge.***

The mean monocyte counts in peripheral blood of the immunised group decreased from day 0 to their lowest on day 3 PC(Figure 7.11) and then increased gradually to the highest on day 12 PC. Analysis for day to day and sheep to sheep differences between the medians monocyte counts in the immunised group revealed significant differences between days (day 3 vs 7) (F=8.80, P<0.05) and a highly significant difference between sheep to sheep (F=31.51, P<0.05) (Table 7.10). The

mean monocyte counts in peripheral blood of the control behaved in a similar manner as those of the immunised group (Figure7.11). Despite the differences between day to day and sheep to sheep of monocyte counts, there were no differences between the median counts of the immunised and control groups ($t=2.093$, $P>0.05$).

The mean neutrophil counts of the immunised group increased from day 0 to the highest on day 3 PC (Figure 7.12) and decreased significantly on day 7 PC ($p<0.05$, Table 7.11) followed by another increase on day 10 PC which restored the counts back to those of day 3 PC. There were however no differences between sheep ($P=0.05$). The mean neutrophil counts of the control group behaved in a similar manner like those of the immunised group (Figure 7.12). In the control group there were no significant differences between days 3 and 7 PC ($F=2.69$, $p>0.05$) and also between sheep ($F=5.18$, $p>0.05$).

Analysis for differences group to group differences showed that there were no differences between them ($t=2.093$, $p=0.286$).

#### *7.3.6.6. Cytotoxicity assays after challenge..*

Cytotoxic activity was not demonstrated with effectors obtained at day 3 and day 7 PC. The LDH OD values of the controls were higher than those of the tests. As a consequence to the high LDH OD values of the controls computation of the percent lysis resulted in negative values.

#### *7.3.6.7. Post mortem findings.*

The PM changes observed in fatal cases of the immunised and control groups were similar to those observed in classical cases of heartwater except for the intense gall

staining observed in tissues adjacent to the liver. A summary of each pathological change is given in Table 7.12.

### 7.3.6.8. Examination of Brain Smears

Examination of Giemsa stained smears prepared from the brains of immunised and control sheep revealed the presence of a high number of *C. ruminantium* in the cytoplasm of the capillary endothelial cells (Figure 7.13). Counts of the number of infected endothelial cells (Ecs) are summarised in Table 7.13.

The mean infection rate of Ecs of the immunised sheep was 30 % (SD±23.3) with a range of 20 to 45.5% while the mean rate of infection in the controls was 62.95% with a range of 43.75 to 77.5%.

Analysis for differences in the degree of infection using the means of infected brain endothelial cells (Ecs) of immunised and control groups showed that the degree of infection of the Ecs in the immunised sheep was significantly lower than those of the controls ((P<0.05).

## 7.4. Discussion.

The work reported above constitutes the first trial of a recombinant antigen for immunisation against *Cowdria ruminantium*. Partial protection in mice, but not in sheep was achieved but in the latter there was significant reduction in the capillary endothelial cell infection rate. The use of capillary infection rate to determine the level of infection is novel and easier than the more technically demanding DNA hybridisation method reported for the same purpose (Mahan, *et al.*, 1992).

Immunisation of mice and sheep with full length recombinant 58kDa Hsp (GroEl) or the 35kDa recombinant protein (subclone of GroEL) led to development of specific antibodies which reacted to the native homologue (58kDa) the EB of *C. ruminantium*. Antibodies to the full length recombinant protein were detected by day 14 post inoculation in sheep, in mice antibodies to the 58kDa and subclone 35kDa antigen were detected on day 21 and 34 post inoculation respectively. Except for four mice immunised with the full length recombinant GroEL the rest did not survive challenge with a dose of $1.0 \times 10^3$ EBs of the Welgevonden stock. Two of 10 mice immunised with bacterial lysate or with *E. coli* carrying the plasmid pBSCr9.4 survived challenge. Sera from these survivors showed strong reactions to the 58kDa antigen in contrast to sera from infection/treatment mice which reacted weakly to the same antigens. There was a significant difference between the survval time of immunised and control mice: the controls lived longer than the immunised ones. This suggests that immunisation with the recombinant antigen induced development of a rapid course of fatal heartwater in the mice. This may be explained by the

activity of pro-inflammatory cytokines such as IL-1 or TNF-α (Delanoy, Lekeux and Miossec, 1993) or IL-6 (Bensaid *et al.*, 1993) which are known to exacerbate certain diseases if high levels are produced for long periods of time during infection.

All the 6 sheep immunised with the recombinant 58kDa Hsp were not protected against challenge with a dose of $3.0 \times 10^5$ EBs of the Welgevonden stock. There was no significant difference in the period of survival or in the lesions observed on post mortem between immunised and control sheep. Different methods of computing the challenge dose have been described, but the determinantion of $LD_{50}$ in small ruminants is required since repeated use of large number of animals cannot be considered humane. The choice of challenge was emperical but the use of EB quantitation allows accuracy to formulate. It is important to determine the incubation period associated with reduced EB challenge, with the aim of getting an incubation period similar to that which occurs with tick infection (about 13-14 day) in the natural disease compared to 6-8 days in this trial. However significant differences between the rates infection of brain capillary endothelial cells of the immunised and control groups were observed. It is possible that the reduction was mediated by antibodies since antibodies from immune sheep inhibit infectivity of *C. ruminantium* to mice in the presence of complement (Du Plessis, 1993). The other possibility is that undetected cellular responses such as the release of IFN-γ by natural killer cells, or by $CD8^+$ and γδ T cells (Kaufmann, 1993) could have been responsible. Low levels of IFN-γ which did not influence the course of the disease but which were able to reduce the infection rates of brain endothelial cells of the immunised sheep may

also be responsible. *In vitro* studies have shown that IFN-γ inhibits infection of endothelial cells and reduces the yield of EBs from infected cultures (Totte *et al.*, 1994; Mahan *et al.*, 1996).

Following immunisation the serological responses of mice and sheep were characterised by higher $IgG_1$ than $IgG_2$ ELISA OD values (Chapter 5.3.8) This suggests that a Th2 response driven by IL-4 was induced by immunisation with recombinant antigens. These findings are in agreement with those of other workers who have demonstrated that immunisation with dead organisms or soluble proteins preferentially stimulates $CD4^+$ Th2 cells and antibody production but usually fails to induce a Th1/$CD8^+$ CTL response (Schirmbeck *et al.*, 1994). However low $IgG_2$ was detectable which suggests that a Th1 type response is also induced but at a lower level.

Determination of the T cell phenotypes in PBMC obtained from immunised sheep then stimulated *in vitro* with recombinant 58kDa revealed the presence of a higher proportion of $CD4^+$ T cells than $CD8^+$ T cells. CD4:CD8 ratios of 3:1 and higher were observed in these cultures. In addition determination of the phenotype of T cells in PBMC obtained from immunised sheep at day 3, 7 and day 10 of challenge revealed high CD4:CD8 ratios. No evidence of a $CD8^+$ response was observed in one I/T and one of the IEB animals (chapter 6) was noted in this experiment. There was an absence of IFN-γ in supernatants from PBMC cultures stimulated with either the 58kDa Hsp or 35kDa subclone *in vitro* which indicates that these antigens may not stimulate release of IFN-γ from PBMC of immunised

animals. In contrast to the proliferative responses to 58kDa antigen of PBMC from goats immunised with IEB or I/T, EBs stimulated PBMC from the same goats immunised to produce IFN-γ *in vitro* (Section 6.3.3).

There was a highly significant decrease in monocyte counts on days 3 compared to day 7 PC ($P < 0.006$) in the immunised group. This suggested that monocytes were actively involved in the immune response against challenge in this group and the decrease may also reflect activation and reduction in migration from circulation from the because of intravascular infection.

Monocytes play a very important role in immune response to intracellular bacteria. They form part of the innate immune system which responds to infection before T cell mediated protection occurs. In addition they process and present antigens to T cells to initiate T cell responses. IFN-γ activates macrophages making them potent effectors of microbicidal CMI following T cell-mediated (Tizard, 1992). IFN-γ was not detected in PBMC culture supernatants in this study. However challenge with virulent *Cowdria* may have induced its production. Macrophages are crucial in inducing Th1 type responses in that they secrete IL-12 after stimulation by certain microbial factors secreted by live bacteria. IL-12 plays a key role in the development of Th1 responses to intracellular bacteria (Cheers and Zhan, 1996). IL-12 stimulates naive $CD4^+$ T cells to secrete IFN-γ which which in turn stimulates B cells to produce $IgG_2$ in the bovine (Estes *et al.,* 1994) mouse and rat (Mossman and Coffman, 1989) and in addition activates both macrophages and neutrophils into powerful effectors to destroy intracellular bacteria (Lukacs *et al.,* 1985, Chiang

Murata and Roth 1991). Evidence from experiments with the intracellular bacterium *Listeria monocytogenes* indicate that killed bacteria do not induce a Th1 response but the addition of IL-12 to killed bacteria stimulates a protective immunity against challenge (Miller *et al.,* 1995*).* Stress proteins fail to induce CMI unless injected with special adjuvants such as ISCOMS (Cheers and Zhan, 1996). In the experiments described in this chapter complete Freund's adjuvant and Mantonide were used to deliver bacterial lysates or purified antigens and judging from the results, these adjuvants potentiated an antibody mediated response but not a cell mediated one.

Lack of proliferation of PBMC from I/T goats stimulated with 35kDa recombinant protein (Section 6.3.3) suggested that this part of GroEL lacks T cell epitopes or absence of detectable cellular responses to the 58kDa may underline the failure to protect the animals. Also since it was purified and soluble it may not have been processed correctly.

Failure of this recombinant to protect sheep and the partial protection of mice mice and against virulent challenge with *C. ruminantium* indicates that the recombinant 58kDa Hsp alone does not induce a strong protective immunity and may therefore require to be administered with other antigens to enhance the partial protection demonstrated in mice. Immunisation with two or more recombinant antigens have been shown to give better protection due to potentiation of the immune response (Shankarapa *et al.*, 1991). It is also possible that this antigen does not play any role in protective immunity to *C. ruminantium* but this would be surprising given

the role of Hsp 60 in other infections (Young *et al.*, 1988). Another possibility is that the challenge dose used was too high, particularly as the virulent Welgevonden stock which causes fatal heartwater was used (Du Plessis and Van Gas, 1989). The most important reason for the failure of protection could be due the inability of this antigen to stimulate PBMC from goats which had been immunised by I/T or with IEBs to produce IFN-γ whereas, EBs stimulated them to produce this cytokine (Section 6.3.3). *In vitro* studies have demonstrated that IFN-γ inhibits infection of endothelial cells by *C. ruminantium* (Totte *et al.,* 1994, Mahan *et al.*, 1996) indicating that this cytokine could be involved in protective immune responses to *C. ruminantium.*

The findings of this study lead to a number of recommendations which include:

1. The relationship between challenge dose of EBs and incubation period and mortality of the Welgevonden stock in small stock needs to be determined for future protection experiments.
2. Other adjuvants and other methods of antigen delivery should be tested to find out if they will improve the immunogenicity of this recombinant antigen.
3. Experiments with full length purified GroEL should be carried out in mice to investigate the value of this antigen in immune responses to *C. ruminantium.*

Table 7.1. Organisation of groups of mice for immunisation with recombinant (Cr9.4) containing 58kDa Hsp (GroEL), bacterial control (pBScript) and adjuvant controls (FCA, IFA).

| Group | No .Mice | Antigen | Adjuvant | dose | Booster | Day Challenged |
|---|---|---|---|---|---|---|
| A | 10 | Cr9.4 | FCA | 200 µg | 100 µg+IFA | 35 PI |
| B | 10 | Cr9.4 | FIA | 200 µg | 100 µg/ml | 35 PI |
| C | 10 | pBScript | FCA | 200 µg | 100 µg/ml | 35 PI |
| D | 10 | pBScript | FIA | 200 µg | 100 µg/ml | 35 PI |
| E | 5 | none | CFA | 0.1 ml | 0.1 ml | 35 PI |
| F | 5 | none | FIA | 0.1 ml | " | 35 PI |

**Key**
Cr9.4 = lysate of pBSCr9.4/JM109 containing the 58kDa recombinant antigen.
pBScript = lysate of *E. coli* containing plasmid pBluesript (pBS/JM109);
FCA = Freund's complete adjuvant; IFA = Freund's incomplete adjuvant
PI = days post inoculation
Booster was given day 14 and 21 PI.

Table 7 2. Immunisation of mice with 35kDa subclone protein of *C. ruminantium.* Groups received purified 35kDa (A, B), bacterial lysate containing 35kDa antigen (C, D), with presence (E, F) and absence of adjuvant (G).

| Group | Antigen | Dose | Adjuvant | Day of Boosters | Day challenged |
|---|---|---|---|---|---|
| A | 35kDa pure | 50 µg | FCA | 14, 21 PI. | 35 PI. |
| B | 35kDa pure | 50 µg | Mantonide | 14, 21 PI. | 35 PI |
| C | 35kDa lysate | 200 µg | FCA | 14, 21 PI. | 35 PI |
| D | 35kDa lysate | 200 µg | Mantonide. | 14, 21 PI. | 35 PI |
| E | FCA | 200 µl | None | 21 PI. | 35 PI |
| F | Mantonide | 200 µl | None | 21 PI | 35 PI |
| G | None | None | '' | None | 35 PI |

**Key**

FCA = Freund's complete adjuvant
PI = days post inoculation

Table 7.3. Results of *Cowdria* challenging Groups of mice which had been immunised with 58kDa Hsp recombinant protein as bacterial lysate (A, B), or whole bacteria (G). Groups C and D are controls for Groups A and B, Groups E and F are controls for adjuvants Group H, is control for Group G.

| Group | Antigen+Adjuvant | Challenge dose | No.challenged | No dead | survivors | Days to death ± SD |
|---|---|---|---|---|---|---|
| A | Cr9.4+FCA | $1.0x10^3$ EBs | 9 | 8 | 1 | 8 ± 1.2 |
| B | Cr9.4+FIA | '' | 10 | 9 | 1 | 9 ± 0.4 |
| C | pBS+FCA | '' | 10 | 10 | 0 | 11 ± 2.5 |
| D | pBS+FIA | '' | 10 | 10 | 0 | 11 ± 1.2 |
| E | FCA | '' | 5 | 5 | 0 | 11 ± 0.5 |
| F | FIA | '' | 5 | 5 | 0 | 10.5 ± 1 |
| G(‡) | E.coli JM109/pBScr9.4 | '' | 10 | 8 | 2 | 8.5 ± 1 |
| H(‡) | E.coli JM109/pBS | '' | 10 | 10 | 0 | 7 ± 0.5 |

**Key**
(‡) mice in group G and H received 5 doses of whole bacteria (Section 7.2.3.2).
Cr9.4 = bacterial lysate containing recombinant 58kDa hsp
pBS = plasmid bluescript
FCA = Freund complete adjuvant
FIA = Freund incomplete adjuvant

Table 7.4. Summary data of analysis for differences between medians of days of survival of groups of mice immunised with 58kDa Hsp recombinant antigen (GpsA, B, C and G) and controls (G and H).

| groups | t value | P value | significance |
|---|---|---|---|
| GpsA vs GpB | 2.365 | 0.503 | NS |
| GpA vs GpC | 2.362 | 0.0148 | S |
| GPA vs GpD | 2.365 | 0.026 | S |
| Gp G vs Gp H | 2.364 | 1.0 | NS |

**Key**
S = Significant, NS= Not significant

Table 7.5. Challenge of mice immunised with 35kDa antigen in purified form (A, B), as lysate (C, D) and controls: Survival data.

| Group | Treatment | No.immunised or inoculated | Days to death | No. survivors $1^0$ ch | $2^0$ ch | Overall survival |
|---|---|---|---|---|---|---|
| A | 35 kDa+ FCA | 5 | 11.7 ± 2.5 | 3/5 | 0/3 | 0/5 |
| B | 35kDa+ Mantonide | 5 | 11.3 ± 1.2 | 4/5 | 0/4 | 0/5 |
| C | Lysate + FCA | 5 | 10 ± 1 | 0/5 | NA | 0/5 |
| D | Lysate +Mantonide. | 5 | 11.5 ± 2.4 | 0/5 | NA | 0/5 |
| E | plasmid +FCA | 5 | 10 ± 1 | 4/5 | 3/4 | 1/5 |
| F | FCA | 4 | 11 ± 1 | 0/5 | NA | 0/5 |
| G | uninoculated | 5 | 10.7 ± 2.4 | 0/5 | NA | 0/5 |

**Key**
$1^0$ & $2^0$ ch = first and second challenge respectively.
FCA = Freund complete adjuvant

Table 7.6. Summary of immunisation and challenge of mice immunised with 58kDa Hsp (Cr9.4), with 35kDa subclone (pure/lysate) and control mice inoculated with plasmid only (pBS), with adjuvants (FCA, FIA) or uninoculated mice.

| Antigen/adjuvant | No.mice | No.dead | No.survivors | Immune to 2nd challenge$\Psi$ |
|---|---|---|---|---|
| Cr9.4 + FCA | 9* | 8 | 1 | 1/1 |
| Cr9.4 + FIA | 10 | 9 | 1 | 1/1 |
| Cr9.4 (whole) | 10 | 8 | 2 | 2/2 |
| 35 kDa + FCA | 5 | 2 | 3 | 0/3 |
| 35kDa + Mantonide | 5 | 5 | 0 | NA |
| 35 kDa Lysate + FCA | 5 | 1 | 4 | 0/4 |
| 35kDa lysate+ Mantonide | 5 | 5 | 0 | NA |
| plasmids+ FCA 15 | 15 | 0 | NA | |
| " + FIA | 10 | 10 | 0 | NA |
| FCA none | 10 | 6 | 4‡ | 1/4 |
| FIA " | 5 | 5 | 0 | NA |
| Uninoculated " | 5 | 5 | 0 | NA |

**Key**

* one of the mice in 10 died during inoculation

‡ 3 out of 4 died on second challenge

$\Psi$ mice survived two more challenges with 2 x $10^3$ EB's of Welgevonden stock.

Summary

| | No. | Dead | survived |
|---|---|---|---|
| Received 58kDa & 35kDa | 49 | 45 | 4 |
| controls | 45 | 44 | 1 |

Table 7.7a. Clinical data from challenge of immunised and control sheep indicating incubation period, febrile response, duration of febrile response and length of time to death.

(a) immunised group n =(6)

| sheep/No | Treatment | I.P | Highest Temp. | Days of fever | Days to death PC |
|---|---|---|---|---|---|
| S66 | GroEL | 7 | 42.2 | 6 | 12 |
| S69 | '' | 7 | 42.2 | 9 | 14 |
| S71 | '' | 7 | 42.3 | 7 | 12 |
| S72 | '' | 7 | 42.0 | 5 | 11 |
| S74 | '' | 7 | 41.7 | 4 | 10 |
| S76 | '' | 6 | 42.2 | 6 | 11 |
| Medians | | 6 | 42.2 | 6 | 11.5 |
| Means±SD | | 6.83±0. | 42±0.2 | 6.2±1.7 | 11.7±1.2 |

(b) control group n = (6)

| sheep/No | Treatment | I.P | Highest Temp. | Days of fever | Days to death PC |
|---|---|---|---|---|---|
| S67 | pBS | 9 | 42.5 | 5 | 13 |
| S68 | '' | 8 | 42.2 | 5 | 12 |
| S70 | '' | 7 | 42.1 | 6 | 12 |
| S73 | '' | 7 | 41.7 | 5 | 11 |
| S75 | '' | 7 | 41.9 | 5 | 11 |
| S77 | '' | 7 | 41.7 | 6 | 11 |
| Medians | | 7 | 42.0 $^{o}$C | 5 | 11.5 |
| Mean ± SD | | 7.5±0.84 | 42.0±0.31 | 5.3±0.52 | 11.7±0.82 |

**Key**

GroEL = lysate containing recombinant 58 kDa Hsp.
pBS = lysate containing plasmid control pBluescript
I.P = Incubation period; D fever = days of fever, p.c. = post challenge
Max. Temp = Maximum temperature reaction of sheep.

Table 7.8. Analysis for differences between median counts of $CD4^+$ T cell of days (day 3 vs day 7 PC), sheep to sheep in the immunised and control groups and groups (immunised vs control).

| | F value | P value | Significance |
|---|---|---|---|
| immunised group = (6) | | | |
| Differences between days PC (day 3 vs 7) | 9.11 | 0.006 | HS |
| Differences between sheep | 9.11 | 0.006 | HS |
| control group n = (6) | | | |
| Differences between days PC (day 3 vs 7) | 16.79 | 0.0006 | HS |
| Differences between sheep | 2.66 | 0.09 | NS |
| Differences between groups | t = 0.99, | p = 0.37 | NS |

**Key**

HS = highly significant
NS = not significant

Table 7.9. Analysis for differences between median counts of $CD8^+$ T cell of days (day 3 vs day 7 PC), sheep to sheep in the immunised and control groups and groups (immunised vs control).

| | F value | P value | Significance |
|---|---|---|---|
| immunised group n= (6) | | | |
| Differences between days PC (day 3 vs 7) | 2.81 | 0.11 | NS |
| Differences between sheep | 3.09 | 0.06 | NS |
| control group n= (6) | | | |
| Differences between days PC (day 3 vs 7) | 2.38 | 0.14 | NS |
| Differences between sheep | 1.95 | 0.17 | NS |
| Differences between groups | t = -0.91 | 0.41 | NS |

Key
NS = not significant.

Table 7.10. Analysis for differences between median counts of monocytes of days (day 3 vs day 7 PC), sheep to sheep in the immunised and control groups and groups (immunised vs control).

| | F value | P value | Significance |
|---|---|---|---|
| immunised group n= (6). | | | |
| Differences between days PC (day 3 vs 7) | 80 | 0.006 | HS |
| Differences between sheep | 31.51 | 0.0001 | HS |
| control group n= (6). | | | |
| Differences between days PC (day 3 vs 7) | 88 | 0.03 | NS |
| Differences between sheep | 3.48 | 0.04 | NS |
| Differences between groups | t= 2.093 p>0.29 | | NS |

**Key**

HS = highly significant
NS = not significant

Table 7.11. Analysis for differences between neutrophils counts of days (day 3 vs day 7 PC), sheep to sheep in the immunised and control groups and groups (immunised vs control).

| | F value | P value | Significance |
|---|---|---|---|
| immunised group n= (6) | | | |
| Differences between days PC (day 3 vs 7) | 1.59 | 0.025 | S |
| Differences between sheep | 3.33 | 0.05 | NS |
| control group= (6) | | | |
| Differences between days PC (day 3 vs 7) | 2.69 | 0.012 | S |
| Differences between sheep | 5.18 | 0.01 | S |
| Differences between groups | t = 2.093 p>0.05). | | |

**Key**

S = significant
NS = not significant

Table 7.12. Number of cases of immunised or control sheep showing specific pathological changes at necropsy.

| Pathological change | immunised group n=6 | Control group n=6 |
|---|---|---|
| Lung oedema and congestion | 6 | 5 |
| pleural fluid | 4 | 3 |
| hydropericardium | 4* | 4 |
| liver enlargement, icterus | 4 | 5 |
| gastro-intestinal haemorrhage | 1 | 4 |
| Kidney haemorrhages | 3 | 3 |

**Key**

* one of the 4 had endocardial/myocardial haemorrhages

Table 7.13. Infection rates of capillary endothelial cells from immunised and control sheep following challenge with virulent *C. ruminantium*

| Group | Sheep | No.infected cells (per 200 Ec) | % infection |
|---|---|---|---|
| a) immunised | | | |
| | S66 | 40 | 20 |
| | S69 | 47 | 23.5 |
| | S71 | 91 | 45.5 |
| | S72 | 52 | 26.0 |
| | S74 | 45 | 22.5 |
| | S76 | 90 | 45 |
| Mean±SD | | 60.8 ± 23.3 | 30% |
| b) controls | | | |
| | S67 | 87.5 | 43.75 |
| | S68 | 119 | 59.5 |
| | S70 | 137 | 68.5 |
| | S73 | 108 | 61.5 |
| | S75 | 155 | 77.5 |
| | S77 | 134 | 67.0 |
| Mean ± SD | | 144.5 ± 23.8 | 62.95% |

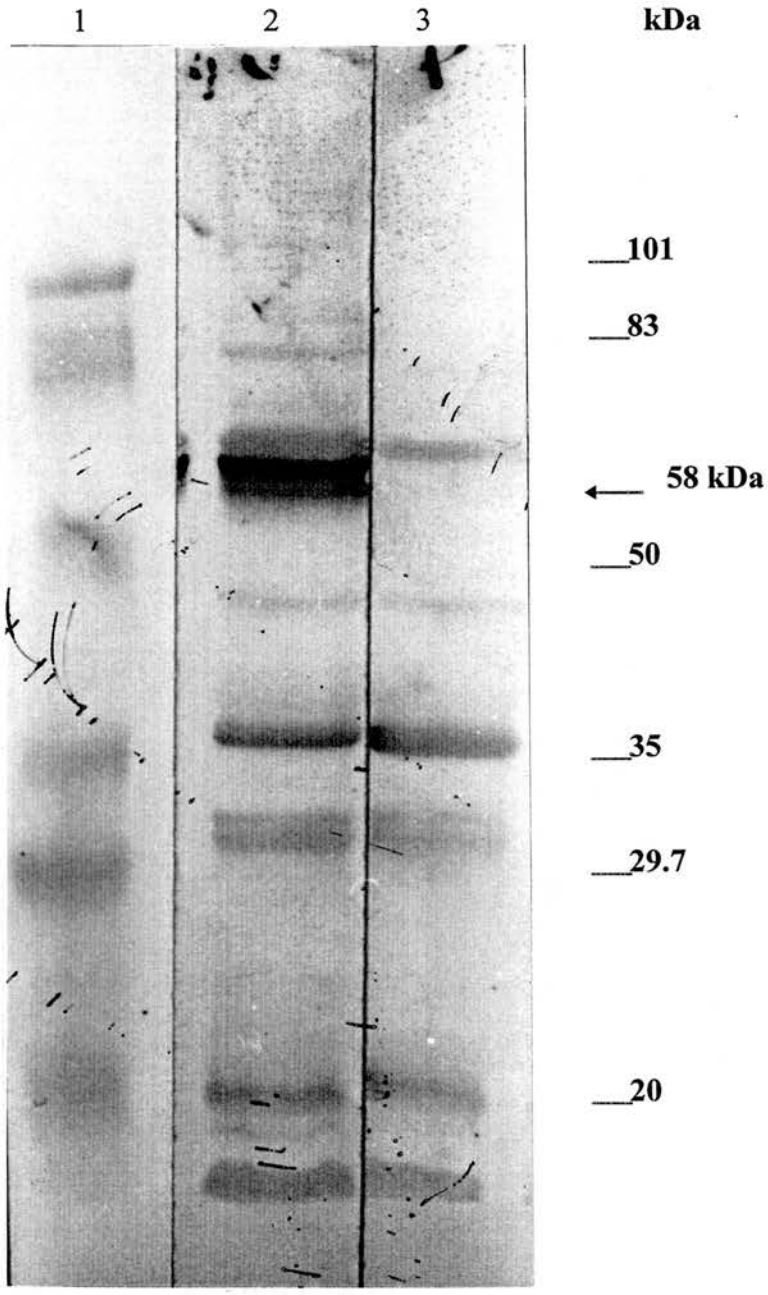



Fig ure 7.1. Western blots of bacterial lysates containing recombinant 58 kDa Hsp demonstrating the presence of recombinant 58kDa Hsp in lysate. Western blots were reacted with sera from an immune goat.
Lane 1, SDS low molecular weight protein standards; lane 2, lysate of JM109/pBSCr9.4 indicating presence of recombinant antigen (58kDa Hsp), thick black band indicated by arrow; lane 3, control plasmid (pBluecript) in *E.coli*.

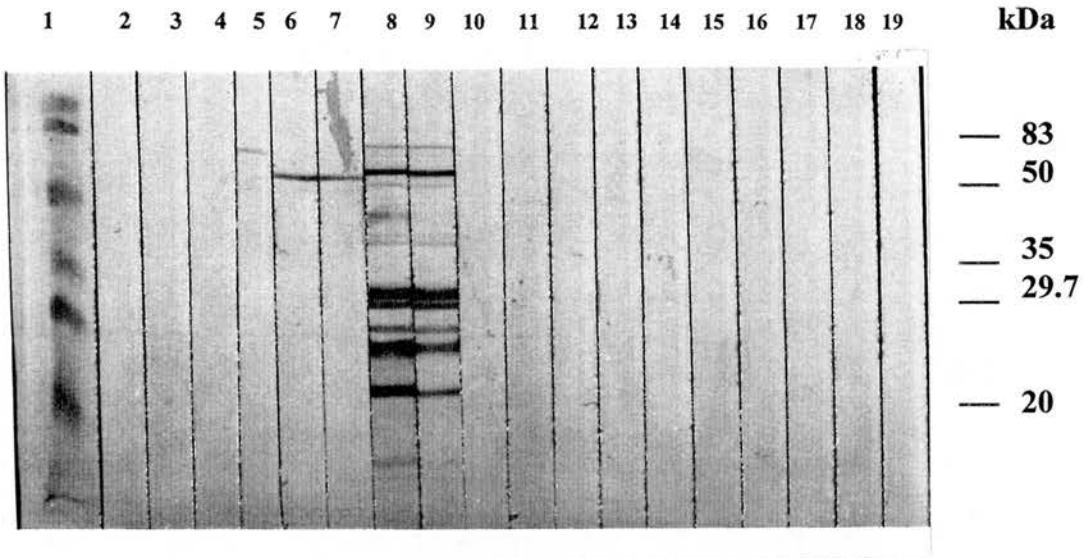



Figure 7.2. Western blots demonstrating recognition of C. *ruminantium* EB antigens by antibodies in pooled sera obtained from mice immunised with 58 kDa Hsp recombinant antigen and absence of such response in the plasmid control group.

Lanes 1, SDS Molecular weight standards; lanes 2 to 7, sequential sera from immunised group which received 58kDa Hsp. Lanes 10 to 14 sequential sera from plasmid control group; lanes 15 to 16, FCA control group (day 21 PI); lanes17 and 18 negative control sera; lanes 8 and 9 positive control sera(day 34 PI) from I/T mouse
lanes 2 and 3, 10 and 11 sera collected pre-immunisation. Lanes 4 and 5, 12 and 13, sera collected day 21 Pilanes 6 and 7 , 14 and 15 serum collected day 28 PI.

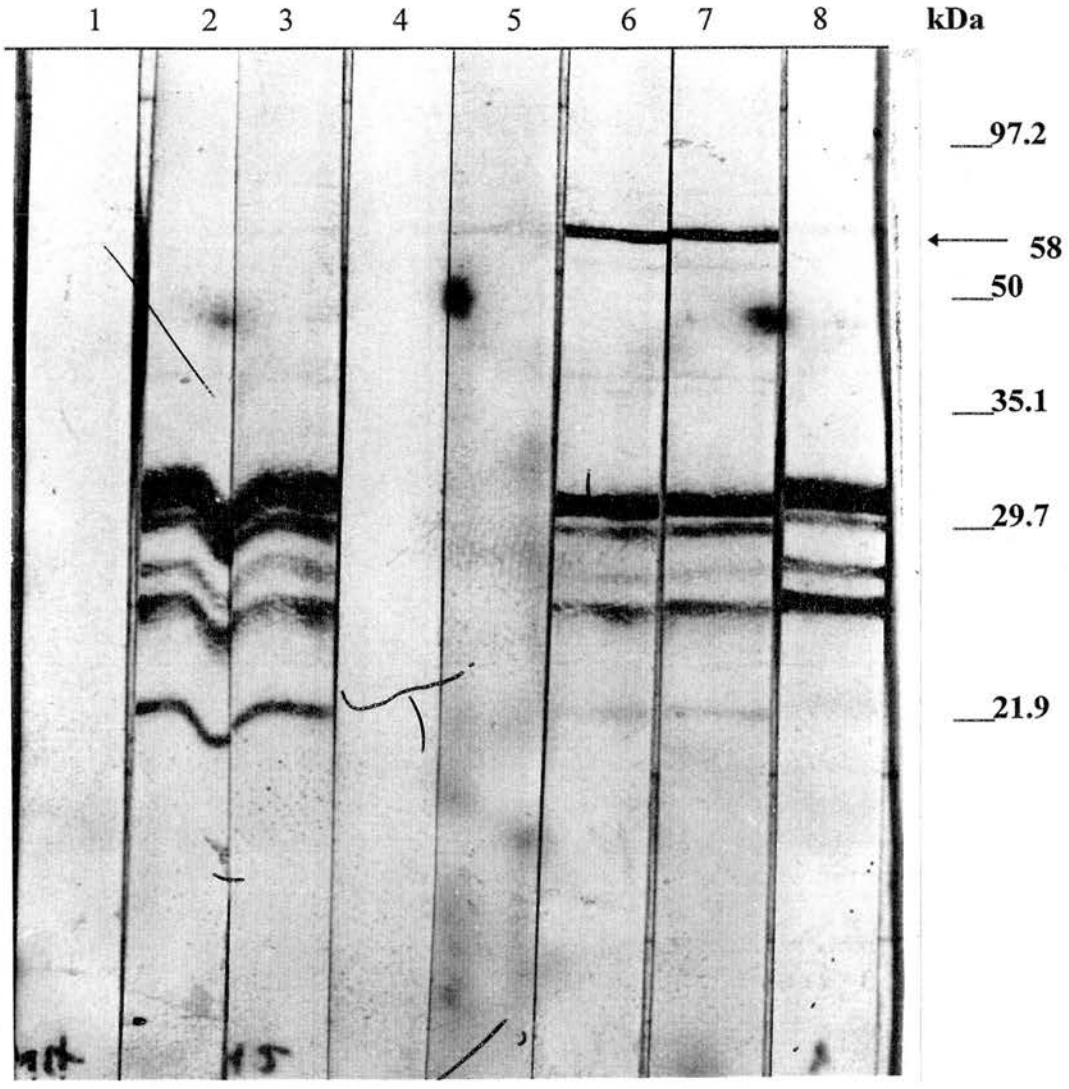



Figure 7.3. Western blots demonstrating presence of specific antibodies to at least 5 antigens of the Cowdria EB following challenge of mice immunised with bacterial lysates containing 58kDa Hsp recombinant antigen.
Lanes were tested as follows: lane 1, negative control serum; lanes 2 and 3, day positive control serum from an I/T mouse; lanes 4 and 5, day 34 PI sera from plasmid control mouse; lanes 6 and 7, day 14 PC sera from mice immunised with 58kDa Hsp and lane 8 was tested with day 34 PC sera from a mouse immunised with 58kDa recombinant rotein ($95A_4$). Arrow indicates the position of 58 kDa antigen.

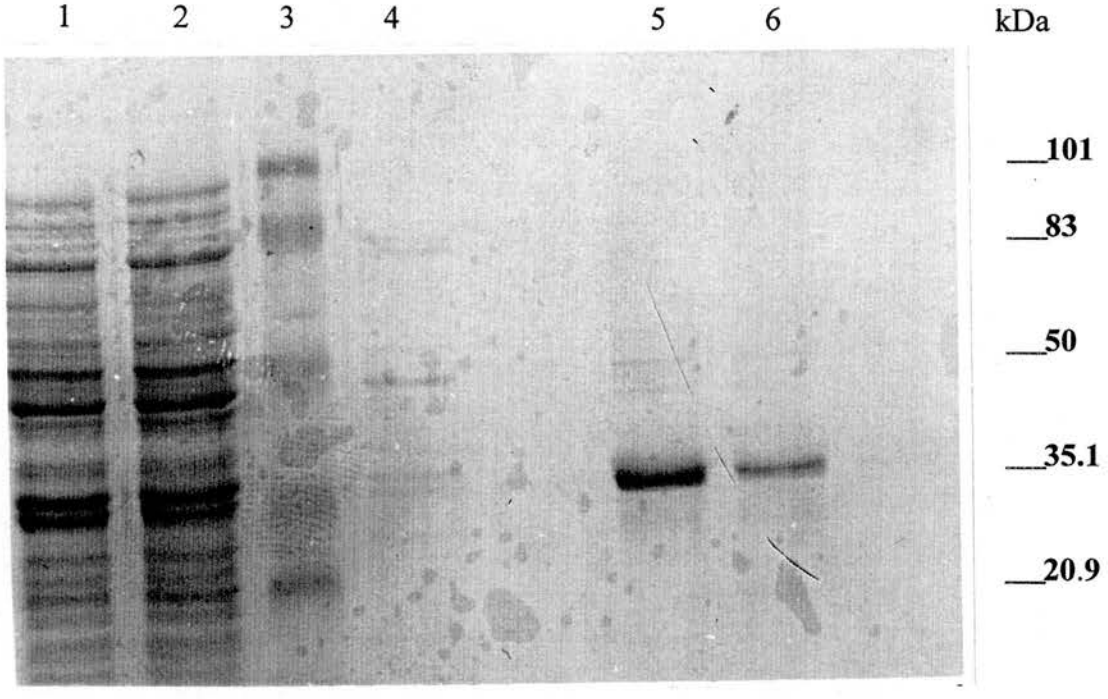



Figure 7.4a. Coomassie blue stained SDS-PAGE gel to demonstrate purity of 35kDa subclone protein antigen after affinity chromatography.
Lanes 1 and 2, un-purified bacterial lysate; Lanes 3, SDS molecular weight markers; Lane 4 wash; Lanes 5 and 6 first and second eluates of purified 35kDa subclone protein antigen.

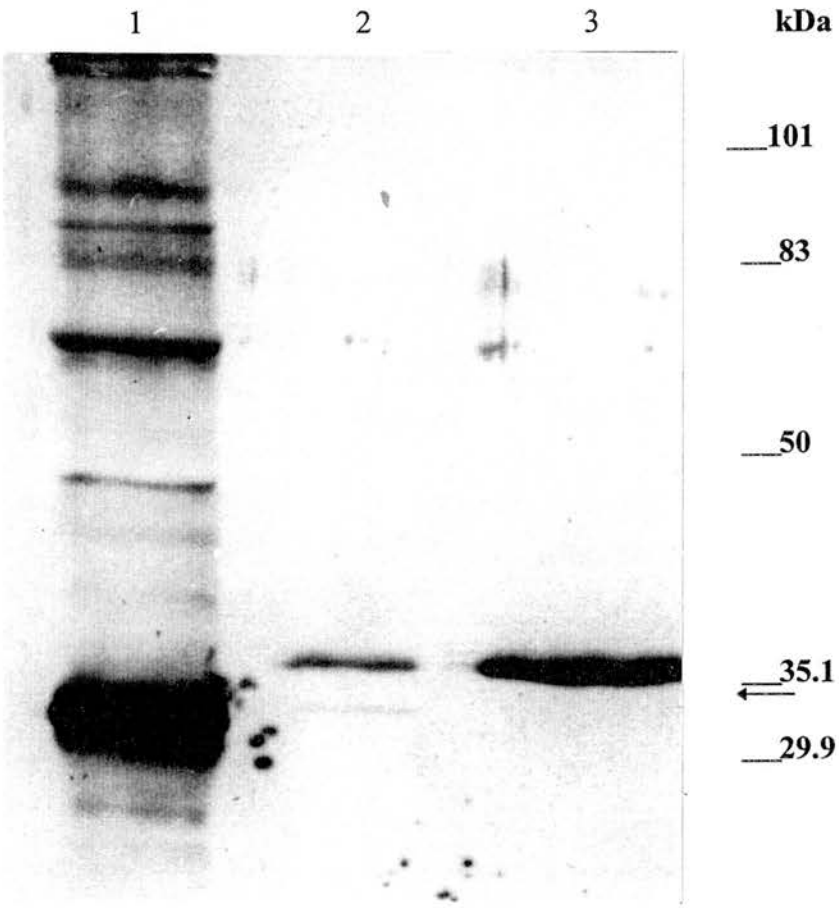



Figure 7.4b. Western blots of *C. ruminantium* EBs and the purified recombinant 35kDa subclone indicating strong reactions between the 35kDa by immune sera obtained at day 28 PI serum from an immune goat.
Lane1, EB antigens; lane 2 and 3, purified 35 kDa subclone protein first and second eluates respectively.

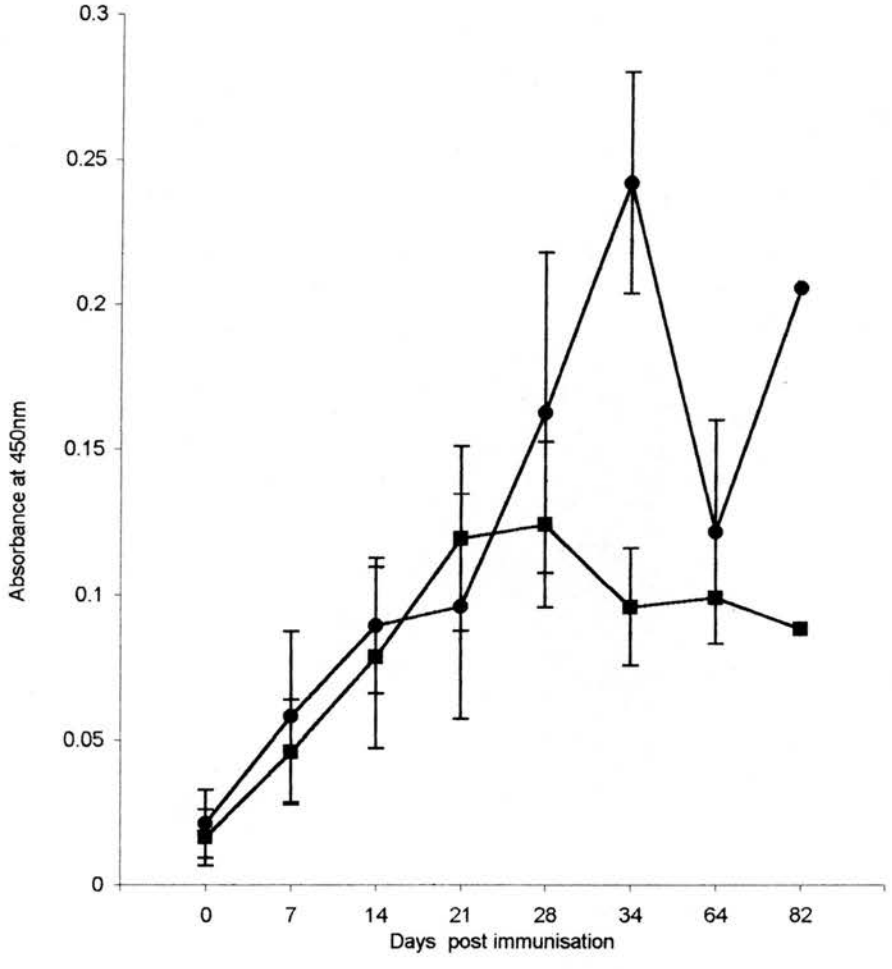



Figure 7.5. Serological responses to 35kDa recombinant antigen of immunised and control sheep.
Antibody responses of sheep immunised with recombinant 58kDa ( ● ) and control sheep inoculated with plasmid only ( ■ ). Results are mean OD of two tests.

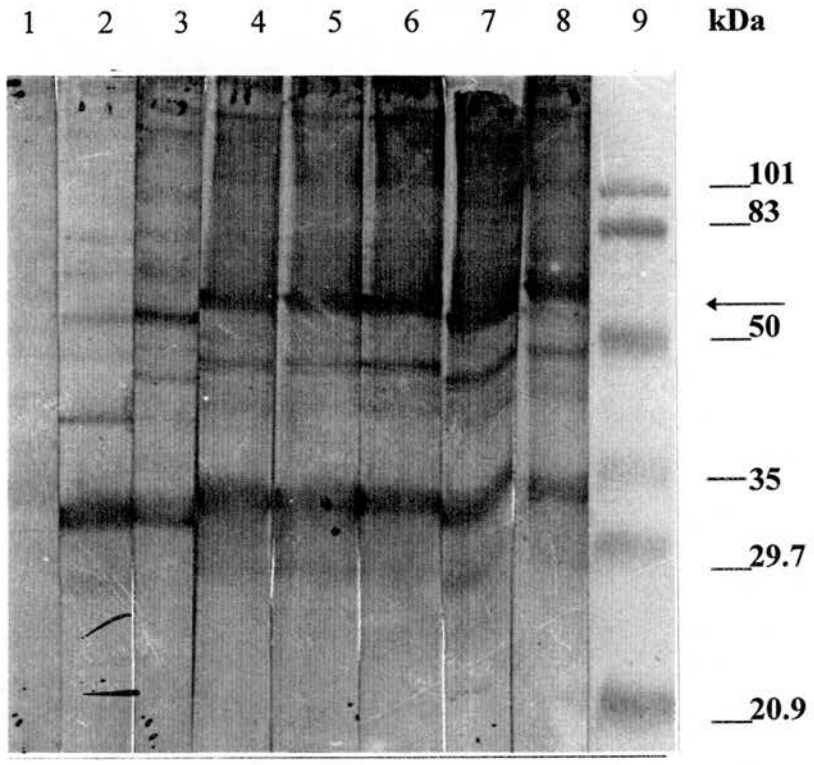



Figure 7.6a. Western blots demonstrating recognition of the 58 kDa EB antigen of *C. ruminantium* by sequential sera from sheep immunised with recombinant 58 kDa Hsp.

Lanes were tested with sera as follows: lane 1, day 0 sera; lane 2, day 14 sera; lane 3, day 21 sera; lane 4, day 28 sera; lane 5 day 34 sera; lane 6 day 64 sera; lane 7, day 82 sera and lane 8 day 143 sera. Lane 9 SDS low molecular weight markers.
Arrow indicates the position of the 58kDa antigen.

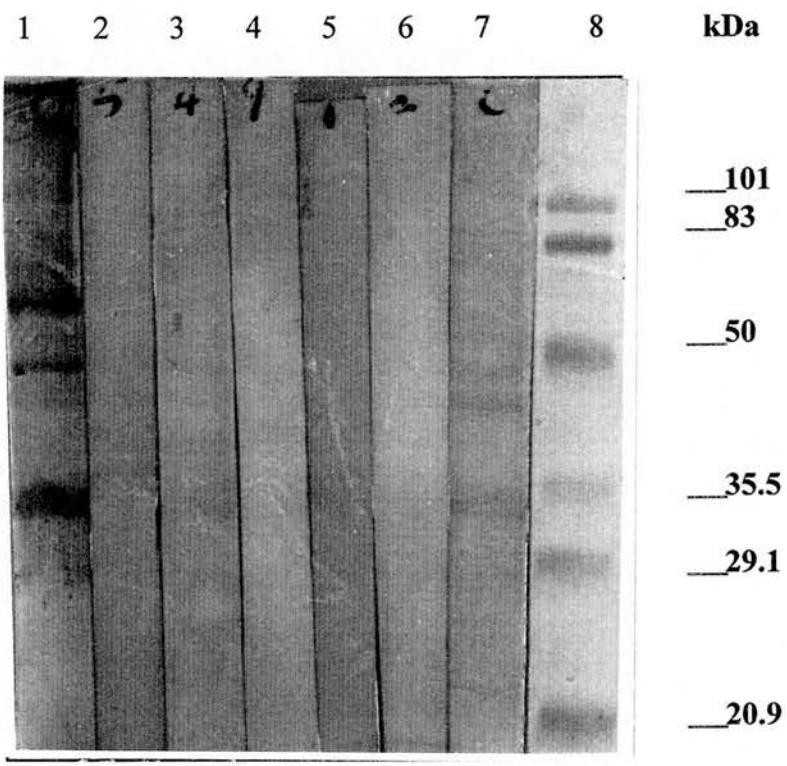



Figure 7.6b. Western blots demonstrating absence of reactions between the 58kDa antigen of the EB with sequential sera from sheep inoculated with plasmid (pBS) control.
Lane 1, day 34 sera from immunised group; lane 2, day 0 serum from control group; lane 3, day 14 serum; lane 4, day 21 serum; lane 5, day 28 serum; lane 6, day 64 serum; lane 7 day 143 serum. Lane 8, SDS low molecular weight standards.

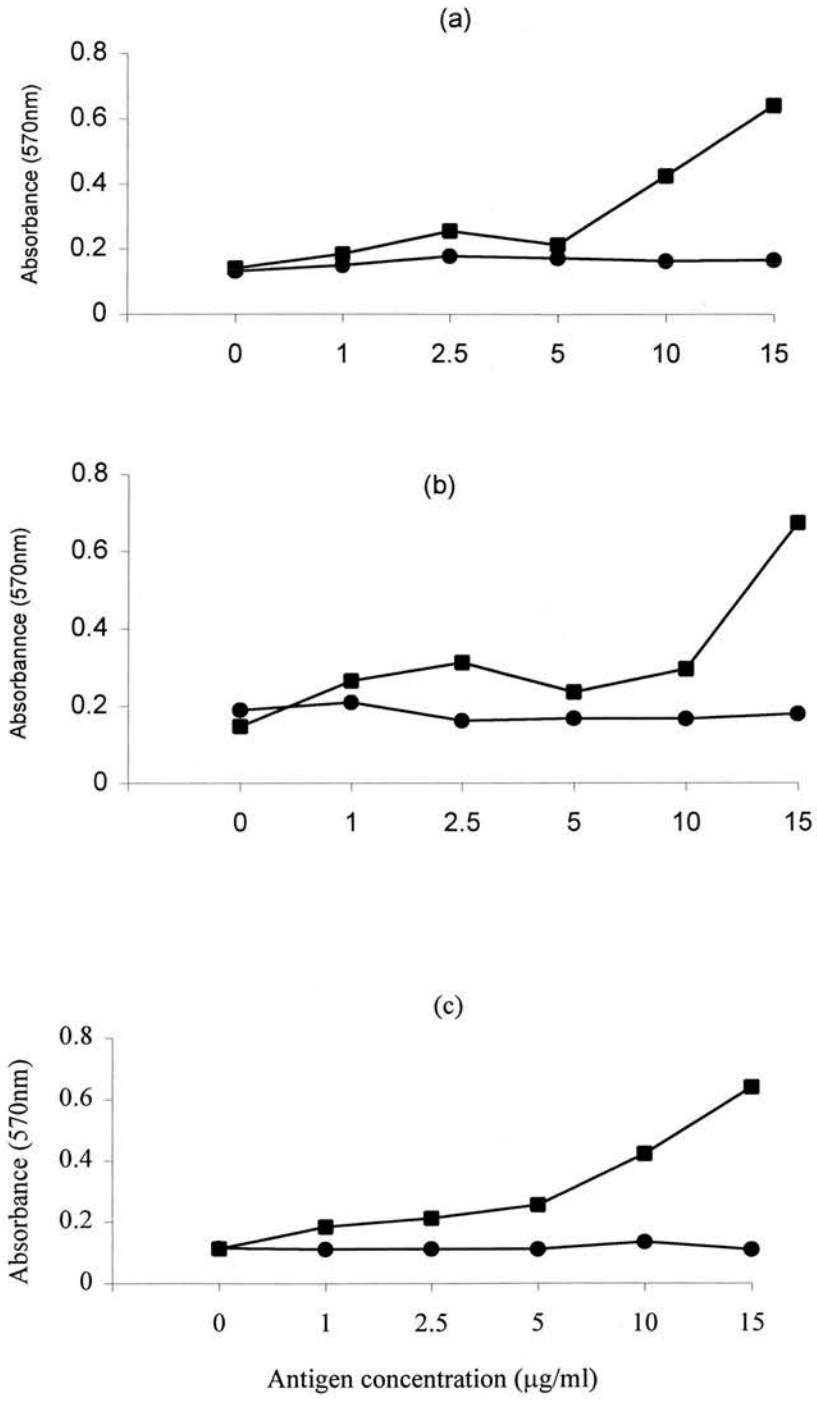



Figure 7.7.Dose dependent proliferative responses of PBMC from two sheep immunised with recombinant 58kDa Hsp : S71 (Figure 7.7a), S72 (Figure 7.7b) and control sheep inoculated with plasmid pBS: S67 (Figure 7.7c). PBMC were stimulated *in vitro* with bacterial lysates of the recombinant 58kDa (Cr9.4) or plasmid (pBS).
Proliferative responses to Cr9.4 ( ■ ), and proliferative responses to plasmid /pBS ( ● )
The OD values represent the median of 3 tests determined by MTS assay.

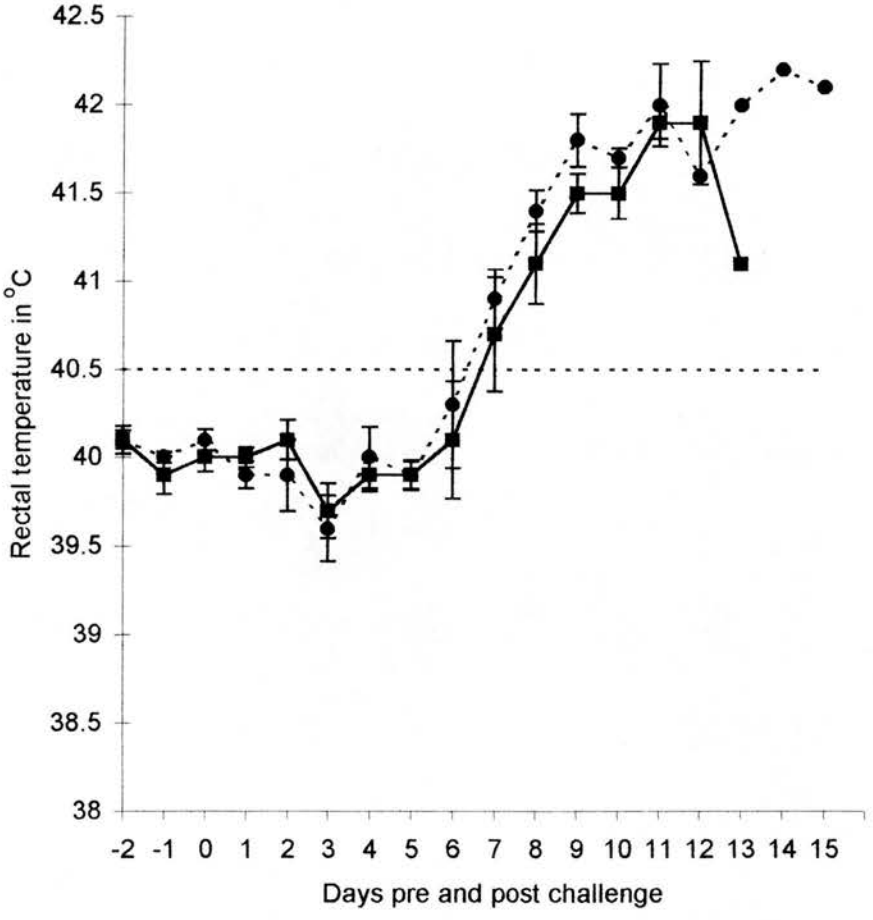



Figure 7.8. Temperature reactions of immunised and control sheep in °C before and after challenge. Mean daily temperatures of immunised group ( ● ) and mean daily of the control group ( ■ ).
The dotted line indicates the maximum upper limit of 'normal' temperature.

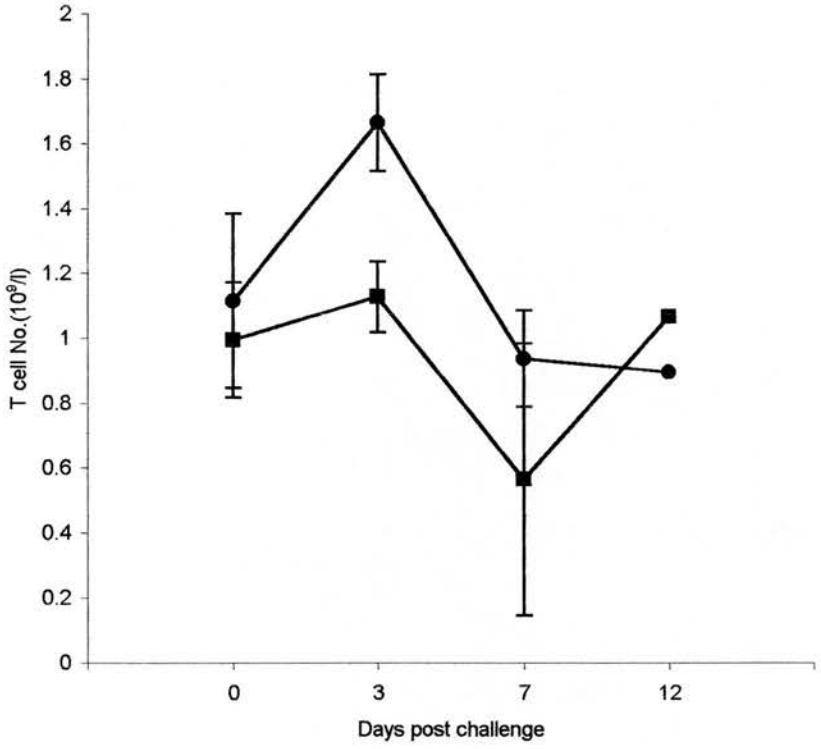



Figure 7.9. Mean counts of $CD4^+$ T cells in peripheral blood of sheep immunised with recombinant 58kDa Hsp and controls before and post challenge.
Mean counts of the immunised group ( ● ), and counts of the control group ( ■ )

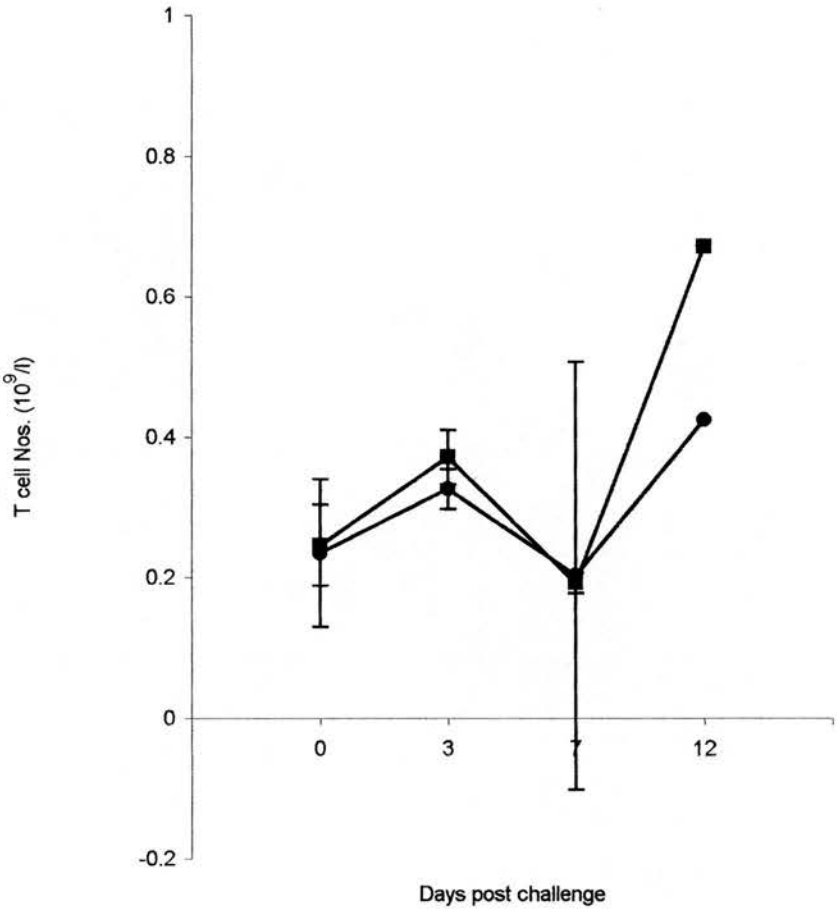



Figure 7.10. Mean counts of $CD8^+$ T cells in peripheral blood of sheep immunised with recombinant 58kDa Hsp and controls before and post challenge.
Mean $CD8^+$ counts of the immunised group ( ● ), Mean counts of the control group ( ■ ).

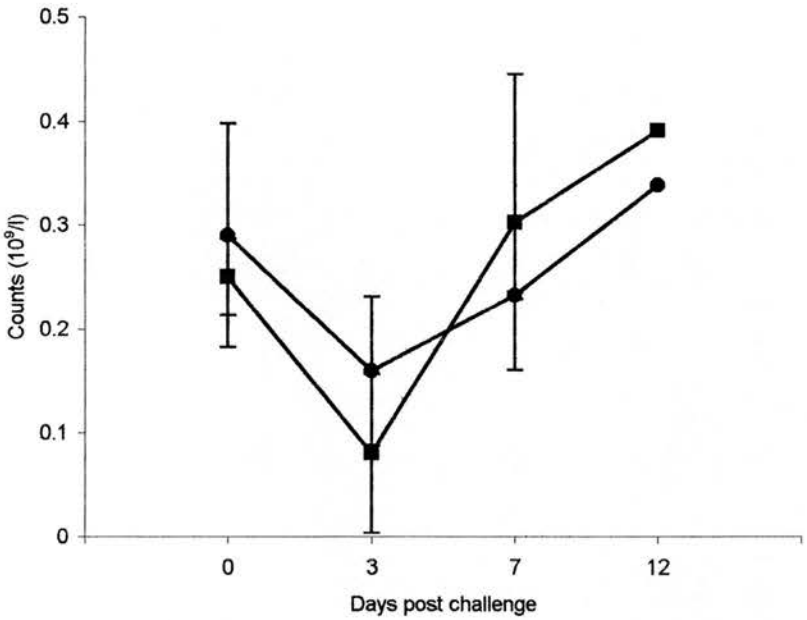



Figure 7.11. Mean monocyte counts in peripheral blood of sheep immunised with recombinant 58 kDa Hsp and control sheep before and post- challenge. Mean counts for group immunised with recombinant 58kDa hsp ( ● ), and counts of the control group ( ■ ).

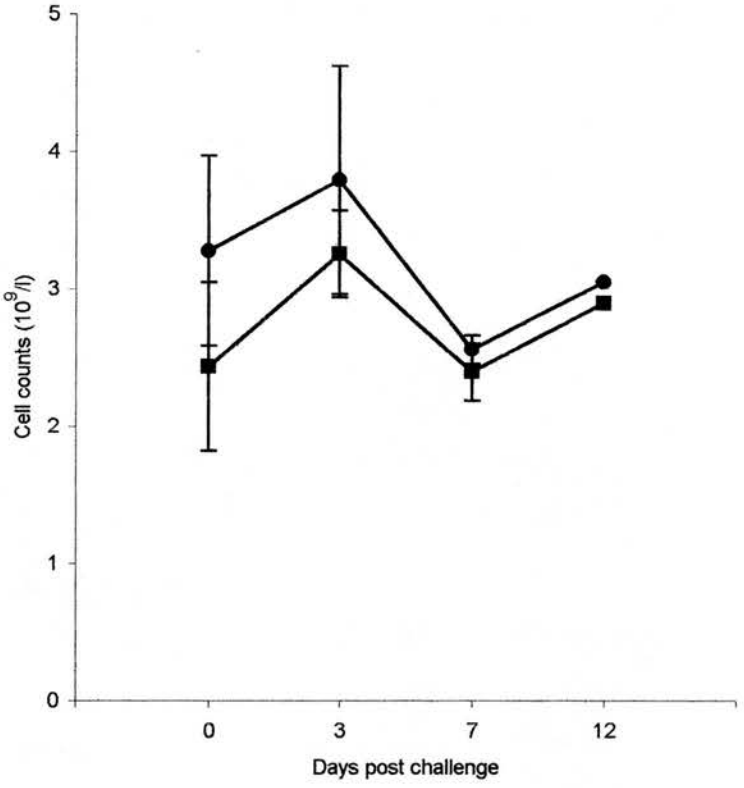



Figure 7.12 . Mean neutrophil counts in peripheral blood of sheep immunised with recombinant 58kDa Hsp and control before and post-challenge.
Mean counts of sheep immunised with recombinant 58kDa hsp ( ● ), and mean counts of the control group ( ■)

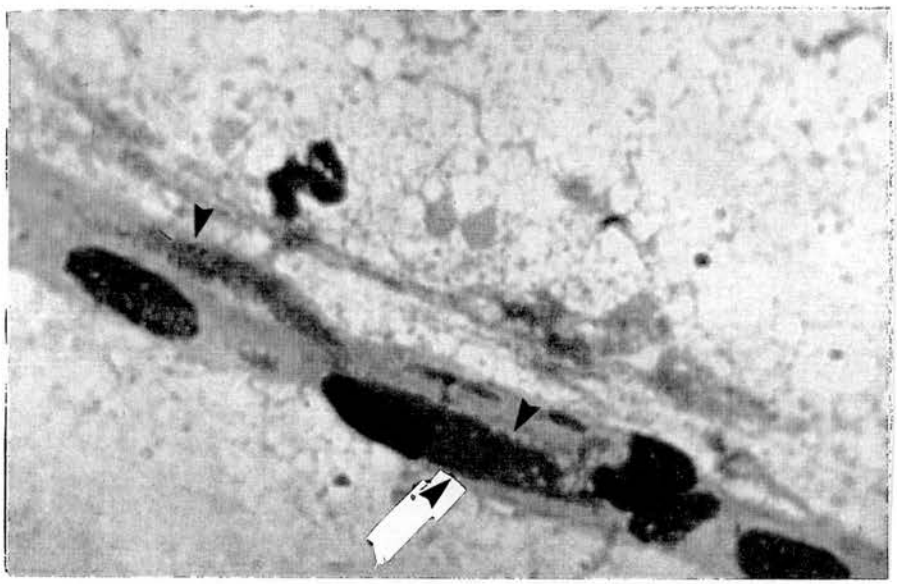


Figure 7.13. Giemsa stained brain smear from challenged sheep showing brain capillary endothelial cells infected with *C. ruminantium*. Arrows indicate elementary bodies.

# CHAPTER EIGHT

## GENERAL DISCUSSION AND CONCLUSIONS

Experiments carried out in the early part of this century by scientists in South Africa produced valuable knowledge about immunity to *C. ruminantium*. The first attempts to protect ruminants against heartwater were carried out by Dixon (1898) followed by Hutcheon (1902), Spreull (1904) and Theiler (1905, 1906). Three discoveries by Alexander (1931), Neitz (1941) and Neitz and Alexander (1941) culminated in the development of a successful infection and treatment (I/T) method for vaccination ruminants which is still in use in South Africa.

Immunisation of ruminants by I/T confers solid immunity to homologous challenge however, this method has some significant limitations including:- the risk of spreading other tick-borne diseases, the requirement of qualified personnel to administer it by the intravenous route, the possibility of direct loss through virulence of infection and the limited scale on which it can be used. These factors have led to the search for safer vaccines. Two types of vaccines have since been developed and tested with promising results in the 1990's. They are an attenuated culture vaccine (Jongejan, 1991; Gueye *et al.*, 1994) and an inactivated elementary body vaccine (Tafesse, 1992; Martinez *et al.,* 1993, 1994; Mahan *et al.,* 1995). However, the mechanisms involved in protective immunity against heartwater in the ruminant have remained poorly understood. In the mouse, protective immunity against heartwater is cell mediated and is mainly by cytotoxic T lymphocytes (Lyt-$2^+$/$CD8^+$ T cells) as demonstrated by experiments in adoptive transfer of $CD8^+$ T cells from immune mice

to naive mice (Du Plessis, 1991, 1992). It was speculated that protective immunity in ruminants was cell-mediated since passive transfer of hyper-immune serum (Alexander, 1931) or injection of gamma globulins from immune sheep into susceptible sheep did not increase protection (Du Plessis, 1970a; Du Plessis and Bezuidenhout, 1979). Additionally elementary bodies were not neutralised *in vitro* by sera from immune animals (Martinez *et al.,* 1993, 1994) however, antibodies neutralised *C. ruminantium* (Byrom *et al.,* 1993; Du Plessis, 1993) *in vitro* which explains the disparity or provide reasons for differences observed and provide evidence for the possible role of antibodies in protective immunity.

In order to understand the mechanisms involved in protective immune responses against heartwater, antigenic components of *C. ruminantium* have to be identified and characterised. Identification of antigens of *C. ruminantium* was carried out first by Jongejan and Thielemans (1989a) who used a combination of SDS-PAGE and immunoblotting to identify an immunodominant 32kDa protein antigen which has cross reactive epitopes with the genus *Ehrlichia.* Further identification of antigenic components was carried out by other workers (Rossouw *et al.*, 1990; Van Kleef *et al.*, 1993; Mahan *et al.*, 1993). These studies have generated valuable knowledge of the antigenic components recognised by sera from immunised animals. However, they have concentrated on antigens in general and not those which are on the surface of the EB. In this study surface components of the EB of *C. ruminantium* (Welgevonden stock) were identified by biotin labelling and were found to be antigenic by immunoblotting. Six major surface antigenic proteins of

21kDa, 28kDa, 31kDa, 62kDa, 74kDa and 115kDa were identified. Three of the antigens identified (21kDa, 32kDa and 58kDa) belong to a group of *C. ruminantium* antigens termed major antigenic peptides (Mahan *et al.*, 1994; Mahan, 1995). SDS-PAGE of individual EB antigens led to the purification of 2 antigens of 24kDa and 32kDa which were used cell proliferation assays using PBMC from immune goats. Although these antigens did not stimulate PBMC to proliferate, their purification paved a way for further investigation into their value in immunisation experiments in the future.

Immunisation of goats with IEBs stimulated development of antibodies to 5 antigens of the EB of molecular masses 24kDa, 27kDa, 28kDa, 32kDa, and 58kDa. After challenge of IEB-immunised goats, those which survived developed antibodies reacting with additional antigens, of 14kDa, 21kDa, 45kDa, 66kDa, and 80kDa. Antibodies to the same EB antigens given above were detected in sera collected from two of the survivors 18 to 24 months after recovery from challenge. Since these animals were protected against virulent challenge, the antigens recognised by sera after challenge with live EBs may be significant in the development of protective immune responses. The appearance of extra bands after challenge may also indicate that these antigens are less abundant and responses to them are made only on replication of *C ruminantium in vivo*. Immunisation of ruminants with the 32kDa (MAP1) antigen did not lead to protection (van Vliet *et al.,* 1994) but it is possible that the method of presentation (oil adjuvant) of the antigen may not have been optimal for iinducing cellular responses because T helper epitopes on MAP1 have

been identified (Totte 1996 personal communication). Other antigens of *C. ruminantium* need to be evaluated to identify those which induce protective immunity against this rickettsia. However, the nature of the protective immune response must be defined in order that suitable markers of immunity can be determined to assist the screening for adjuvants and antigens.

Immunisation with IEB appears to prime the immune system to respond with a Th1 type response. The evidence for this was production of $IgG_2$ and protection of goats against challenge, as shown by the responses of 4 IEB-immuised goats which survived challenge. Upon challenge, of the IEB immunised goats they developed a temperature reaction, temporary loss of appetite and mild depression for some days followed by recovery in others. This indicated that the type of immunity which develops after immunisation with IEBs seems to be insufficient to prevent severe cowdriosis if the level of challenge is high, in contrast to challenge of I/T immunised animals which show few or no clinical signs.

The immune response to challenge by IEB-immunised goats seems to be determined by the dose of the challenge inoculum since IEB-immunised sheep exposed to natural field conditions survived (Mahan *et al.*, 1995). Experimental challenge of goats led to development of temperature reaction and other clinical siigns of heartwater with some of them not surviving (Martinez *et al.*, 1993; Tafesse, 1992). In this study IEB-immunised goats and sheep immunised with a 58kDa recombinant Hsp antigen developed severe heartwater and did not survive challenge. In contrast immunisation of ruminants by I/T confers solid immunity against

challenge and no development of clinical signs (Uilenberg *et al.*, 1983). The I/T method of immunisation leads to a predominant $IgG_1$ response, an indication that live *C. ruminantium* does not induce a classical Th1 response but rather surprisingly induces a Th2 antibody profile. This could be due to the induction of IL-4 or IL-10, cytokines which promote $IgG_1$, IgM and IgE production by B cells (Tizard, 1995). Bovine IL-4 upreglates productionof $IgG_1$, IgM and IgGE but not $IgG_2$ (Estes *et al.*, 1995) and *Ehrlichia chaffeensis* induces expression of IL-10 by human macrophages a cytokine which has similar effects to IL-4 (Lee and Rikihisa, 1996). Despite the fact that a Th2 type antibody response is induced, goats which recover from infection after treatment are protected against subsequent challenge. Development of high $CD8^+$ counts very late after challenge suggests that the antibody profile is perhaps misleading and may not reflect the cellular bias. The I/T method is used extensively in South Africa where up to 250,000 doses are used every year (Bezuidenhout *et al.*, 1994). The mechanisms by which *C. ruminantium* circumvents the immune system resulting in long-term carrier status are not known.

In this study results of a preliminary experiment carried out to determine the effects of live and inactivated EBs on the proliferative responses of PBMC from an I/T goat showed that live EBs reduced the proliferative responses. This result suggests that *C. ruminantium* down regulates lymphocyte responses or the antigen presenting capacity of macrophages possibly by inhibiting phagolysosome fusion as observed in *Chlamydia psittaci* (Wyrick and Brownridge, 1978). However, Du Plessis *et al.*, (1992) argues that in mice phagolysosomal fusion occurs in

macrophages which leads to $CD8^+$ responses because antigens are processed by the cytoplasmic (MHC class I) route. It could also be due to down regulation of MHC class II expression (Totte *et al.,* 1997) or co-stimulatory molecule expression.

The IgG concentration of sera from cattle exposed to natural challenge of heartwater were similar to those of I/T cattle and goats, indicating that ruminants respond to natural and experimental *C. ruminantium* infection by producing mainly $IgG_1$ (Th2 type response). In contrast, I/T recovered mice develop a dominant $IgG_2$ response, an indication of a classical Th1 response which is consistent with the development of antigen specific CTL important in the control of infection (Du Plessis et al 1992). The differences between the immune responses of ruminants and mice questions the validity of using mice for protection experiments against *C. ruminantium.*

It is now known that immune responses to infections of intracellular organisms are determined at the beginning of infection by the type of cytokine induced by the invading organism (Kaufmann, 1995). Organisms which are potent inducers of IL-12 such as *Listeria monocytogenes* induce secretion of IL-12 from macrophages early in the infection leading to IFN-γ production by NK cells which favours development of a Th1 type response. In contrast, organisms which are potent inducers of IL-4 such as *Leishmania major,* will induce a Th2 type response. In this study the IgG isotype responses of sera from naturally and experimentally infected cattle and those of I/T goats were characterised by dominant $IgG_1$ antibody responses. An explanation for this could be that *C. ruminantium* induces IL-4 or IL-

10 production at the outset of infection, to reduce the anti-rickettsial effects of IL-12 and IFN-γ, and stimulation of B cell responses (by IL-10) towards $IgG_1$ (Th2) type response. Evidence from studies with a closely related organism, the human pathogen *Ehrlichia chaffeensis* indicates that killed and live ehrlichiae induce expression of mRNA for IL-1β, IL-8 and IL10 by human macrophages and that this could be used by ehrlichiae as a way of delaying development of protective immune responses (Lee and Rikihisa, 1996). It is likely that *C. ruminantium* behaves in a similar manner.

Stimulation of PBMC from goats immunised either by I/T or with IEBs with homologous EBs led to IFN-γ production *in vitro*. This indicates that the immunisation had primed the PBMC to release this cytokine since PBMC from a naive control did not produce IFN-γ after stimulation with EBs. Flow cytometric analysis of PBMC following EB stimulation showed that the proportion of $CD4^+$ increased in contrast to $CD8^+$ T cells indicating that a T helper responses were induced. Challenge of an I/T led to development of a late CTL response (day 17 PC) evidenced by a 5 fold increase in $CD8^+$ counts.

The role played by antibodies in protective immunity against heartwater is controversial. However, natural infection, I/T and IEB immunisation lead to strong $IgG_1$ responses. Adoptive transfer of serum or purified antibodies did not protect mice against challenge (Byrom *et al.*, 1993). In contrast, Du Plessis (1993) found that transfer of serum with complement inhibited infectivity of *C. ruminantium* (Kumm stock) in outbred mice. In this study, sheep immunised with recombinant

58kDa Hsp antigen of *C. ruminantium* developed a dominant $IgG_1$ response and upon challenge they had significantly lower rates of infection in brain capillary endothelial cells than the control group. This suggests that either antibodies had a role perhaps by opsonisation or undetected cellular responses were responsible through IFN-γ released at a low level, to an extent which reduced *Cowdria* infection in brain EC. In view of the unclear role played by antibodies in protective immunity against heartwater, further investigation is necessary to determine the significance of $IgG_1$ and $IgG_2$ in ruminants, as a marker for underlying responses and in terms of their possible roles in protection.

Protective immunity in ruminants is induced by I/T immunisation (Neitz and Alexander, 1941), immunisation with attenuated organisms (Jongejan, 1991; Gueye *et al.*, 1994) and immunisation with inactivated EBs (Martinez *et al.*, 1993, 1994; Mahan *et al.*, 1995). The fact that the IEB vaccine induces a protective immunity is important because it is safe (non-virulent) to use and can be used safely outside the area of geographical origin of the isolate. Inactivated EB vaccines are the best options for protecting ruminants against heartwater in the near future until subunit, recombinant or nucleic acid vaccines are available. In this study immunisation of mice and sheep with a recombinant 58kDa Hsp and a 35kDa subclone was attempted with limited protection against challenge in mice but none in sheep. The fact that only three adjuvants (FCA, FIA and Mantonide ISA50) were used to deliver the recombinant antigens requires that other adjuvants and delivery systems need to be used in protection experiments with this antigen in order to identify a suitable one for

use in future. In addition other recombinant antigens such as MAP1 (van Vliet *et al.*, 1994) and MAP2 (Mahan *et al.,* 1994) and some of the other surface antigens identified in this study (Chapter 4) should be used in protection experiments to identify those which are protective.

Heartwater remains an economically important disease of ruminants in sub-Saharan Africa and the search for a safe and economically feasible vaccine and investigations of the biology of the rickettsia, mechanisms of immunity and pathogenesis should continue.

Finally this study has demonstrated that:

1. Six antigenic proteins of 21, 28, 31, 62 and 72kDa are located on the surface of EBs and are recognised by immune sera from goats immunised with live and inactivated EBs additionally sera obtained from IEB goats before challenge recognised fewer antigens than sera obtained after challenge.
2. Natural and experimental infection of ruminants with *C. ruminantium* results in a Th2 type antibody profile whereas, immunisation of goats and cattle with killed EBs induces a mixed a Th2 profile in goats but Th1/Th2 profile in cattle. Upon challenge of the IEB immunised animals both antibody isotypes are raised, with greater increase in Th1 type antibodies being evident.
3. Mice IgG antibody profiles after infection/treatment are dominated by $IgG_2a$, evidence of a Th1 type response. In contrast, inactivated EBs or recombinant antigen immunisation induces a strong $IgG_1$ antibody responses consistent with a mainly Th2 typeresponse.

4. PBMC from immunised goats are primed to produce IFN-γ upon subsequent stimulation by homologous EBs *in vitro*. The $CD4^+$ and $CD8^+$ T cell responses of immunised goats are dominated by $CD4^+$ for the first 10 day of challenge and after that $CD8^+$ T cells increase to about 5 times that of $CD4^+$ by day 17 PC. $CD8^+$ remained 2 times higher than $CD4^+$ for at least one month.

5. Immunisation of mice with recombinant 58kDa (GroEL) and 35kDa subclone partially protected mice against virulent homologous challenge.

6. Evidence was found that *Cowdria* affects the nature of immune responses in a manner that may facilitate parasite survival and transmission by delaying protective responses.

# REFERENCES


Abdel Rahim, A.M. and Shomein A.M. (1978). Heamatological studies in goats experimentally infected with *Rickettsia ruminantium. Bulletin of Animal Health and Production in Africa,*. **26**:232-235.

Abinanti, F.R. and Marmion, B.P. (1957). Protective or neutralising antibody in Q-fever. *American Journal of. Hygiene*, **66:**173-195.

Afonso A. Langa (1995). Development of antigen immunoassays for the detection of *Cowdria ruminantium,* MSc thesis, University of Edinburgh.

Alexander, R.A. (1931). Heartwater. The present state of our knowledge. *17th Report of the Director of Veterinary Services and Animal Industry*, **1**:89-150.

Alexander, R.A., Neitz, W.O. and Adelaar, T.F. (1946). Heartwater. *Farming in South Africa*, **21**:548-552.

Andreasen, M.P. (1974). Multiplication of *Cowdria ruminantium* in monolayer of tick cells. *Acta Patholologie Microbiology Scandinavica Section B*, **82**: 445-456.

Andrew, H.R. and Norval R.A.I. (1989a). The role of the male of the bont tick (*Amblyomma herbraum*) in the transmission of *Cowdria ruminantium* (heartwater). *Veterinary Parasitology*, **34**:15-23.

Andrew, H.R. and Norval R.A.I. (1989b). The carrier status of sheep and African buffalo recovered from heartwater. *Veterinary Parasitology*, **34**:261-266.

Arnold, R.M. and Asselberg, M. (1981). Tick-borne diseases immunisation of cattle imported into Mozambique. *World Animal Review*, **40**: 23-29.

Balozet, L. (1935). Fievres exanthematiques et heart-water. *Bulletin Acadamie Medicine*, **114**:418-421.

Balozet, L. (1936). Contribution a letude experimentale de la heartwater. *Bulletin Acadamie Veterinaire*, France 1938, **11**:319-323.

Barbet, A.F., Semu, S.M., Chigagure, N., Kelly, P.J., Jongejan, F. and Mahan, S.M. (1994). Size variation of the major immunodominant protein of *Cowdria ruminantium. Clinical Diagnosis and Laboratory Immunology*, **1**:744-746.

Barre, N. and Camus E. (1987). The reservoir status of goats recovered from heartwater. *Onderstepoort Journal of Veterinary Research*, **54**:435-437.

Beaman, L. and Wisseman, Jr., C.L. (1976). Mechanisms of immunity in typhus infection. VI. Differential opsonizing and neutralisation action of human typhus rickettsia-specific cytophilic antibodies in cultures of human macrophages. *Infection and Immunity,* **14**:1071-1076.

Bensaid, A., Bourdoulous, S., Lerhun, D., Calvez, D., Droogman, L. and Martinez, D. (1993). Interleukin-6 expression upon infection by *Cowdria ruminantium* of bovine endothelial cells. *Revue 'd'Elevue et de Medecine Veterinaire des Pays Tropicaux*, **46**: 195.

Bezuidenhout, J.D. (1981). The development of a new heartwater vaccine using nymphae infected with *Cowdria ruminantium. Proceedings of International Congress on Tick Biology and Control* (Eds. G.B. Whitehead and J.D. Gibson), Rhodes University, Grahamstown, pp. 41-45.

Bezuidenhout, J.D. (1982). Diseases of the central nervous system of cattle under tropical conditions. *Proceedings of the 12th World Congress of Cattle,* Amsterdam, The Netherlands.

Bezuidenhout, J.D. (1984). Demonstration of *Cowdria ruminantium* in *Amblyomma hebraeum* by fluorescent antibody, technique, light and electron microscopy. *Onderstepoort Journal of Veterinary Research,* **51**:213-215.

Bezuidenhout, J.D. (1985). The epizootiology and control of heartwater and other tick-borne diseases of cattle in South Africa. *Onderstepoort Journal of Veterinary Research*, **52**:213-215.

Bezuidenhout, J.D. and Spickett, A..M. (1985). An *in vivo* comparison of the efficacy of the heartwater blood and ground-up tick suspension vaccines in calves. *Onderstepoort Journal of Veterinary Research*, **52**:269-271.

Bezuidenhout, J.D., Paterson. C.L. Camilla, and Barnard, B.J.H. (1985). *In vitro* cultivation of *Cowdria ruminantium. Onderstepoort Journal of Veterinary Research*, **52**:113-120.

Bezuidenhout, J.D. and Jacobsz, G.J. (1986). Proof of transovarian transmission of *Cowdria ruminantium* by *Amblyomma variegatum. Onderstepoort Journal of Veterinary Research*, **53**:31-34.

Bezuidenhout, J.D. (1987a). The present state of *Cowdria ruminantium* cultivation in cell lines. *Onderstepoort Journal of Veterinary Research*, **54**:267-269.

Bezuidenhout, J.D. (1987b). Natural transmission of heartwater. *Onderstepoort Jounal of Veterinary Research*, **54**:349-351.

Bezuidenhout, J.D. and Bigalke R.D. (1987). The control of heartwater by means of tick control. *Onderstepoort Journal of Veterinary Research,* **54**:525-528.

Bezuidenhout, J.D., Oliver J.A., Gruss, B. and Badenhorst J.V. (1987). The efficacy of alternative routes for the infection and vaccination of animals with *Cowdria ruminantium. Onderstepoort Journal of Veterinary Research*, **54**:497-506.

Bezuidenhout, J.D. (1989). *Cowdria* vaccines. In *Veterinary Protozoan and Haemoparasite Vaccines* (Editor I.G. Wright,.). CRC Press, Boca Raton, Florida, 31-42pp.

Bezuidenhout, J.D., Prozesky, L., Du Plessis, J.L. and van Amstel, S.R. (1994). Heartwater In *Infectious Diseases of Livestock with Special Reference to Southern Africa,* Vol. I (edited by J.W.A. Coetzer, G.R. Thomson), R.C. Tustin Oxford University Press, 351-370pp.

Bird, P. Reyburn, H.T., Blacklaws, B.A., Allen, D., Nettleton, P., Yirrel, D.L., Watt, N., Sargan, D. and McConell. (1995). The restricted $IgG_1$ antibody response to Maedi-Visna virus following infection but not immunisation with recombinant gag protein. *Clinical and Experimental Immunology,* **102**:274-280.

Birnie, E.F., Burridge, M.J., Camus E and Barre, N. (1985). Heartwater in the Caribbean: Isolation of *Cowdria ruminantium* from Antigua. *Veterinary Record*, **116:**121-123.

Bovarnick, M.R. and Synder, J.C. (1949). Respiration of typhus rickettsiae. *Journal of Experimental Medicine,* **89**:561-565.

Brander, C.,Wss-Coray, Mauri, D., Bettens, F. and Picher, W.J. (1993). Carrier-mediated uptake and presentation of a major histocompatibility complex class I-restricted peptide. *European Journal of Immunology,* **23**:3217-3223.

Brown, C.C., Logan L.L., Mebus C.A and Nagorski, K. (1989). Protection of goats against Caribbean and African heartwater isolates by Ball 3 heartwater vaccine. *Tropical Animal Health and Production*, **21**:00-106.

Brownridge, E.A. and Wyrick, P.B. (1979). Interaction of *Chlamydia psittaci* reticulate bodies with mouse macrophages. *Infection and Immunity*, **24**:697-700.

Butler, J.E., Seawright, G.I., McGiven, P.L. and Gilsdorf, M. (1981). Class and subclass antibody responses of *B. abortus* strain 19-vaccinated and field strain-challenged cattle: evidence for predominant $IgG_1$ responses in infected animals. *Advances in Experimental Medicine and Biology*, **137**:790-791.

Byrne, G.I., Krueger D.A. (1983). Lymphokine-mediated inhibition of chlamydia, replication in mouse fibroblasts is neutralised by anti-gamma interferon immunoglobulin. *Infection and immunity*, **42**:1152-1158.

Byrne G.I.,Grubbs, B., Dickey T.H., Schachter, J. and Williams D.M. (1987). Interferon in recovery from pneumonia due to *Chlamydial trachomatis* in the mouse. *Journal of Infectious Diseases,* **156**:993-996.

Byrne, G.I. and Turco J. (1988). Interferon and none viral pathogens. In G.I Byrne and J. Turco (Editors), Immunology Series Vol. 42. Marcel Dekker, New York, pp 317.

Byrom, B. and Yunker, C.E. (1990). Improved culture conditions for *Cowdria ruminantium* (Rickettsiales), the agent of heartwater disease of ruminants. *Cytotechnology*, **4**:285-290.

Byrom, B., Yunker, C.E., Donovan, P.L. and Smith, G.E. (1991). *In vitro* cultivation of *Cowdria ruminantium* from plasma of infected animals. *Veterinary Microbiology*, **26**:263-268.

Byrom, B., Mahan, S.P. and Barbet, A.F. (1993). The development of antibody to *Cowdria ruminantium* in mice and its role in heartwater disease. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **46**:197-201.

Camus, E. and Barre, N. (1982). La cowdriose (heartwater). Revue generale des connaissance. *Istitut d'Elavage de Medecines, Veterinaire des Pays Tropicaux*, No.4. IMEVT Maisons-Alfort. 147 pp

Camus, E. and Barre, N. (1987). Epidemiology of heartwater in Guadeloupe and in the Caribbean. Onderstepoort. *Journal of Veterinary Research,* **54**:419-426.

Camus, E. and Barre, N. (1988). Heartwater: A Review. OIE Paris, 1988.

Camus, E. and Barre, N. (1990). A*mblyomma variegatum* and associated diseases in the Carbbean: strategies for control and eradication in Guadeloupe. *In Proceedngs of the FAO Expert Consultation on Revision of Strategies of Tick-borne Diseases*, Rome, 25-29 September 1989. *Parassitologia*, **32**:185-194.

Camus, E. (1992). Carrier status in creole cattle and goats after recovery from *Cowdria ruminantium* infection in Guadeloupe. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **45**:133-135.

Camus, E., Barre, Martinez, D., N. and Uilenberg, G. (1996). Heartwater: A Review, OIE Paris, 1996.

Cavallion, J.M. (1993). Cytokines et inflammation In *Les Cytokines*. Masson Paris, 341-358pp.

Chabeuf, N. (1976). Note sur un noyau de bovins francais frisons pie noirs importes au Centre de recherche zootechnique (CRZ). F.J.B. Randriambeloma de Kianjasoa (Madagascar). *Revue Elevage et de Medecine Veterinaire Pays des Tropicaux*, **29**:259-266.

Cheers, C. and Zhan, Y. (1996). How do macrophages distinquish the living from the dead. *Trends in Microbiology*, **4:**453-455

Chiang, Y.W., Murata H. and Roth, J.A. (1991). Activation of bovine neutrophils by recombinant bovine tumor necrosis factor alpha. *Veterinary Immunology and Immunopathology*, **29**:329

Clark, R. (1962). The pathological phsiology of heartwater [*Cowdria* (Rickettsia) *ruminantium* Cowdry, 1926]. *Journal of South African Veterinary Medical Association*, **33**:183-191.

Cowdry, E.V. (1925a). Studies on the aetiology of heartwater I: Observations of a Rickettsia *Rickettsia ruminantium* (N.S.P) in the tissues of the infected animals. *Journal of Experimental Medicine*, **42**:231-252.

Cowdry, E.V. (1925b). Studies on the aetiology of heartwater II: Observations of a Rickettsia, *Rickettsia ruminantium* in the tissues of ticks transmitting the disease. *Journal of Experimental Medicine*, **42**: 253-254.

Cowdry, E.V. (1926). Cytological studies on heatwater. 1. The observation of *Rickettsia ruminantium* in tissues of infected animsls. 11-12th Report of the Director of Veterinary Education and Research, 161-177.

Curasson, M.G. (1932). Le malaides du betail au Soudan francais. *Recuel Medicine Veterinaire Exotique*, **5**:100-110.

Curasson, M.G. and Deply, L. (1928). La heartwater au Soudan francais. *Bulletin Acadamie Veterinaire France*, **81**:231-244.

Dardiri, A.H., Logan, L.L. and Mebus C.A. (1987). Susceptibility of white tailed deer to experimental heartwater infection. *Journal of Wildlife Diseases*, **23**:215-219.

Daubney, R. (1929-1930a). Heartwater (*Rickettsia ruminantium*). Report of Department of Agriculture Kenya, 325-332pp.

Daubney, R. (1930b). Natural transmission of heartwater in sheep by *Amblyomma variegatum* (Fabricus, 1794). *Parasitology*, **22**:260-267

Delanoy, I., Lekeux, P. and Miossec, P. (1993). Cytokines and anticytokine strategies in inflammatory reaction modulation, *Veterinary Research,* **24**:449-467.

Dinarello, C.A. (1993). Modalities for reducing interleukin-1 activity in disease. *Trends in Pharmacological Science*, **14**:155-158.

Dixon, R.W. (1898). Heartwater experiments. *Agricultural Journal of Cape of Good Hope*, **12**:754-762. Cited by Provost and Bezuideenhout (1987)

Du Plessis, J.L. (1970a). Immunity in heartwater I. A preliminary note on the role of antibodies. *Onderstepoort Journal of Veterinary Research*, **37**:147-150.

Du Plessis, J.L. (1970b). Pathogenesis of heartwater. I. *Cowdria ruminantium* in the lymph nodes of domestic animals. *Onderstepoort Journal of Veterinary Research*, **37:**89-96.

Du Plessis, J.L and Kumm, N.A.L. (1971). The passage of *Cowdria ruminantium* in mice. *Journal of South African Veterinary Medical Association,* **42**:217-221.

Du Plessis, J.L. (1975a). Electron microscopy of *Cowdria ruminantium* in the infected reticuloendothelial cells of the mammalin host. *Onderstepoort Journal of Veterinary Research*, **42**:1-14.

Du Plessis, J.L. (1975b). Histopathological studies on the pathogenesis of heartwater as manifested in mice with a strain of *Cowdria ruminantium*. M. Med. vet. thesis University of Pretoria.

Du Plessis, J.L. (1979). Investigation of the natural and acquired resistance of cattle to artificial infection with *Cowdria ruminantium*. *Journal of South African Veterinary Medical Association*, **50**:333-338.

Du Plessis, J.L. and Bezuidenhout, J.D. (1979). Investigation on the natural and acquired resistance of cattle to arftificial infection of *Cowdria ruminantium*. *Journal of the South African Veterinary Medical Association,* **50**:334-338.

Du Plessis, J.L. (1981). The application of indirect immunofluorescent antibody test to the serology of heartwater. *Proceedings of the International Conference on Tick Biology and Control*. Grahamstown, South Africa, 1981, 47-52pp.

Du Plessis, J.L. (1982). Mice infected with a *Cowdria ruminantium*-like agent as a model in study of heartwater. DVS Thesis, University of Pretoria, South Africa, 157pp.

Du Plessis, J.L., Jansen, B.C. and Prozesky L.(1983). Heartwater in Angora goats I. Immunity subsequent to artificial challenge and treatment. *Onderstepoort Journal Veternary Research*, **50**:137-143.

Du Plessis, J.L. (1984). Pathogenicity of tick-borne strains of *Cowdria ruminantium* to mice. *Onderstepoort Journal of Veterinary Research,* **52**:55-61.

Du Plessis, J.L., Bezuidenhout, J.D. and Ludemann C.J.F. (1984). The immunisation of calves against heartwater: Subsequent immmunity both in the absence and presence of natural tick challenge. *Onderstepoort Journal of Veterinary Research*, **51**:193-196.

Du Plessis, J.L. (1985a). A method of determining the *Cowdria ruminantium* infection rate of *Amblyomma herbraeum*: effects in mice injected with tick homogenate. *Onderstepoort Journal of Veterinary Research*, **52**:55-61.

Du Plessis, J.L. (1985b). The natural resistance of cattle to artificial infection with *Cowdria ruminantium* the role of conglutinin. *Onderstepoort Journal of Veterinary Research*, **52**:273-277.

Du Plessis, J.L. and Malan, L. (1987a). The non-specific resistance of cattle to heartwater. *Onderstepoort Journal of Veterinary Research,* **54**:333-338.

Du Plessis, J.L. and Malan, L. (1987b). The apllication of indirect fluorescent antibody test in research on heartwater. *Onderstepoort Journal of Veterinary Research,* **54**:319-325.

Du Plessis, J.L., Malan, L. and Kowalski, Z.E. (1987). The pathogenesis of heartwater *Onderstepoort Journal of Veterinary Research*, **54**:313-318.

Du Plessis J.L. and Malan, L. (1988). Susceptibility of calves born to non-immune cows. *Onderstepoort Journal of Veterinary Research*, **55**:235-237.

Du Plessis, J.L. and Van Gas L. (1989). Immunity of tick exposed seronegative and seropositive small stock challenged with two stocks of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **56**:185-188.

Du Plessis, J.L.,Van Gas L., Olivier J., and Bezuidenhout, J.D. (1989). The heterogenicity of *Cowdria ruminantium* stocks. Cross-immunity and serology in sheep and pathogenicity in mice. *Onderstepoort Journal of Veterinary Research*, **56**:195-201.

Du Plessis, J.L., Potgieter F.T. and Van Gas L. (1990a). An attempt to improve the immunisation of sheep against heartwater by using different combinations of 3 stocks of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **57**:205-208.

Du Plessis, J.L.,Meyers, E.H.H. and van Gas L. (1990b). An attempt to establish an inbred line of mice genetically resisitant to *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research,* **57**:283-285.

Du Plessis, J.L., Berche, P. and Van Gas L. (1991). T cell mediated immunity to *Cowdria ruminantium* in mice the protective role of $Lyt2^+$ T cells. *Onderstepoort Journal of Veterinary Research*, **58:**171-179.

Du Plessis, J.L., Gray, C. and van Strijp, M.F. (1992). Flow cytometric analysis of T cell responses in mice infected with *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **59:**337-338.

Du Plessis, J.L. (1993). An *in vitro* test to demonstrate the inhibitory effect of homogous immune serum on infectivity of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **60**:69-73.

Du Plessis, J.L., Bezuidenhout, J.D., Brett, M.S., Camus, E., Jongejan, F., Mahan, S.M., and Martinez, D. (1993). The sero-diagnosis of heartwater: a comparison of five tests. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **46**:123-129.

Du Plessis, J.L., Boersema, B.R. and van Strijp, M.F. (1994). The detection of cross-reacting with *Cowdria ruminantium* in the sera of domestic ruminants in regions of South Africa where *Amblyomma herbraeum* does not occur. *Onderstepoort Journal of Veterinary Research*, **61**:277-281.

Duriex, V.M. (1931). L'ame'lioration de race bovine malgache. These, Docteur veterinaire, Lyon, 110 pp (cited by Camus and Barre 1988).

Dutta, S.K., Shankarapa, B. and Matingly-Napier, B.L. (1991). Molecular cloning and analysis of recombinant major antigens of *Ehrlichia risticii*. *Infection and Immunity*, **59**:1162-1169.

Edington, A. (1898). Heartwater. *Agricultural Journal of Cape of Good Hope*, **12**:749-754.(cited by Bezuidenhout *et al.* 1994)

Endris, R.G., Haslett, T.M., Birnie, E.F. and Hess, W.R. (1989). A mouse lethal detection and titration of *Cowdia rumunantium* (Kwanyanga strain) in goats and ticks. *Veterinary Microbiology*, **19**:151-165.

Estes, D.M., Closser, N.M. and Allen, G.K. (1994). IFN-γ stimulates production from bovine B cells costimulated with anti-μ and mitogen. *Cellular Immunology*, **154**:287-295.

Estes, D.M., Hirano, A., Heussler, V.T.Dobbelaere, D.A.E. and Brown, W.C. (1995). Expression and biological activities of bovine Interleukin 4: Effects of recombinant bovine interleukin 4 on T cell proliferation and B cell differentiation and proliferation *in vitro*. *Cellular Immunology,* **163**:268-273.

Everlith, K.M., Brooksalder, B. and Splitter, G.A. (1991). Interleukin-alpha induction by *Brucella abortus* LPS vs proteins and modulation with interferon alpha. *Federation of American Societies for Experimental Biology Journal,* **5**:A1349. Meeting Abstract.

Ewing, S.A. (1969). Canine Ehrlichiosis. *Advances in Veterinary Science,* **13**:331-335.

FAO, (1984). Methods of immunisation against *Cowdria ruminantium*. In *Ticks and Ttick-borne Disease Control*. A practical field manual. Volume II. Tick-borne disease control, Rome: FAO, pp. 564-576.

F.A.O (1990). Production Yearbook, **44**:191-192.

Feng', H., Popov, V.L. and Walker, D.H. (1994). Depletion of gamma interferon and tumor necrosis factor alpha in mice with *Rickettsia conorii*-infected endothelium. Impairement of rickettsial nitric oxide production resulting in fatal overwhelming disease. *Infection and Immunity*, **62**:1952-1960.

Fitch, F.W. (1986). T cell clones and T cell receptors. *Microbiological Reviews*, **50**:50-69.

Flasch, E.J., Woodford, J.D, Morzaria, S.P., Dolan, T.T., Shabwana, I. (1990). Indetification of *Babesia bovis* and *Cowdria ruminantium* on the Island of Unguja Zanzibar. *Veterinary Record*, **126**:57-59.

Friis, R.R. (1972). Interaction of cells with *Chlamydia psittaci*. Entry of the parasite and host responses to development. *Journal of Bacteriology,* **110**:706-721.

Gambril, M.R. and Wisemann, Jr. C.L. (1973). Mechanisms of immunity in typus infections. III. Influence of human immune serum and complement on the fate of *Rickettsia mooseri* within human macrophages. *Infection and Immunity*, **8**:631-640.

Garfin, D.E. (1990). One dimensional gel electrophoresis. In *Methods in Enzymology*, **182**:425-441.

Gerros, T.C., Semrad, S.D., Proctor, R.A., and LabBorde, A. (1993). Effects of dose and method of administration of endotoxin on cell mediator release in neonatal calves. *American Journal of Veterinary Research*, **54**:2121-2127.

Grencis, R.K. (1990). Antigen uptake processing and presenation. In *Parasite Immunology,* pp. 63-85 (Editor Behnke J.M), Taylor and Francis Limited.

Grenwal, A.S. and Rouse, B.T. (1979). Characterisation of bovine leukocytes involved in antibody dependent cell cytotoxicity. *International Allergy Applied Immunology*, **60**:169-177.

Gueye, A., Mbengue, M., Kebe B. and Diouf, A. (1982). Note epizootiologie sur la cowdriose bovine dans les Niayes du Senegal. *Revue. d'Elevage et de Medicine veterinaire des Pays Tropicaux*, **35**:212-219.

Gueye, A. Jongejan, F., Mbengue, M., Diouf, A. and Uilenberg, G. (1994). Field trials of an attenuated vaccine against heartwater. *Revue d'Elevage Medecine Veterinaire des Pays Tropicaux*, **47**:401-404.

Hahn, H. and Kaufmann, S.H.E. (1981). The role of cell mediated immunity in bacterial infections. *Reviews in Infectious Diseases,* **3**:1212-1250.

Haig, D.A. (1952). Tick-borne rickettsiosis in South Africa. *Advances in Veterinary Science*, **2**:307-325.

Haig, D.A., Alexander, R.A and Weiss, K.E. (1954). Treatment of heartwater with terramycine. *Journal of South African Veterinary Medical Association,* **25**:45-48.

Hanson, B. (1991). Susceptibility of *Rikettsia tsutsugamushi* Gillian to gamma interferon in cultured mouse cells. *Infection and Immunity,* **59**:4125-4133.

Harding, C.L. and Rui Song (1994). Phagocytic processing of exogenous particulate antigens by macrophages for presentation by Class I MHC molecules. *Infection and Immunity,* **153**:4925-4933.

Hebert, J.I. (1979). Mineral oil adjuvant and the immunisation of laboratory animal. *Handbook of Experimental Immunology* Vol II (Edited by D.M. Weir). Blackwell Scientific Publication, Oxford, pp. A3.1-A3.14.

Hein, W.R. and Mackay, C.R. (1991). Prominence of γ/δ T cells in the rumuniant immune system. *Immunolgy Today*, **12**:30-34

Henning, M.W. (1956). Animal diseases of South Africa, 3rd Ed. South African Central News Agency Ltd. Onderstepoort, 115-1178 (cited by Bezuidenhout *et al.*, 1994).

Howard, C.J., Taylor, G. and Brownlee, J.(1980). Surface receptors for immunoglobulins on polymorphonuclear neutrophils and macrophages. *Research in Veterinary Science,* **29:**128-135.

Howard, C.J. and Morrisson, W.I. (1994). The leukocyte markers, tissue distribution and function. In *Cell Mediated Immunity in Ruminants* pp. (Editors. Bruno Goddeeris, and Ivan Morrison), CRC, Press Inc.pp1-17.

Howell, C.J., De Vos A.J., Bezuidenhout J.D., Portgieter F.T. and Barrowman P.R. (1981). The role of chemical tick eradication in the control of tick-transmitted diseases of cattle. In *Proc. International Conference on Tick Biology and Control*. Grahamstown, South Africa, 61-66.

Hudson, J.R. and Henderson, R.M.(1941). Some preliminary experiments on the survival of heartwater virus in rats. *Journal South African Veterinary Medical Association*, **12**:39:-49.

Hutcheon, D. (1900). History of heartwater. *Agricultural Journal Cape of Good Hope*, **17**:410-417 (cited by Bezuidenhout *et al.*, 1994).

Hutcheon, D. (1902). Heartwater in sheep and goats *Agricultural Journal Cape of Good Hope,* **20**:633-641 (cited by Bezuidenhout *et al.*, 1994).

Ilemobade, A.A. (1976). Study of heartwater and the causative agent *Cowdria ruminantium* (Cowdry, 1925) in Nigeria. Ph.D. Thesis, Ahmadu Bello University Zaria, June 1976.

Ilemobade, A.A. (1978). The persistence of *Cowdria ruminantium* in the blood of recovered anmals. *Tropical Animal Health and Production,* **10**:170.

Ilemobade, A.A. and Blotkamp, C. (1978a). Clinico-pathological study on heartwater in goats. *Tropenmedizin und Parasitologie*, **29**:71-76.

Ilemobade, A.A. and Blotkamp J. (1978b). Heartwater in Nigeria . II Isolation of *Cowdria ruminantium* from live and dead animals and the importance of route of inoculation. *Tropical Animal Health and Production* **10:**39-44

Ilemobade, A.A. and Leeflang P. (1978c).Experiments on the transmission of Cowdria ruminantium by the tick Amblyomma variegatm. In Proceedings of International Conference on Tick-borne diseases and their vectors . Edinburgh, 27 September-1 October 1976, 149-155.

Ingalls, R.R., Rice, P.C., Quereshi, N., Takayama, K., Lin, J.S. and Golenbock, D.T. (1995). The inflammatory cytokines responses to *Chamydia trachomatis* is endotoxin mediated. *Infection and Immunity*, **63**:3125-3130.

Jackson, C. and Neitz, W.O. (1932). On the aetiology of heartwater.. In *18th Report of the Directoer of Veterinary Services and Animal Indusry, South Africa*, 161-173 (cited by Bezuidenhout *et al.*, 1994).

Jerrels, T.R., Turco, J., Winkler, H.H. and Spitlany, G.L. (1986) Neutralisation of lymphokine-mediated anti-rickettsial activity of fibroblasts and macrophages with monoclonal antibody specific for murine interferon gamma. *Infection and Immunity,* **51**:355-359.

Jongejan, F. van Winkelhoff, F.A. and Uilenberg, G. (1980). *Cowdria ruminantium* (Rickettsiaeles) in primary goat kidney cell cultures. *Research in Veterinary Science,* **29**:392-393.

Jongejan, F., Morzaria, S.P., Shariff O.A and Abdalla H.M. (1984). Isolation and transmission of *Cowdria ruminantium* (causal agent of heartwater disease) in Blue Nile Province, Sudan. *Veterinary Research Communications*, **8**:141-145.

Jongejan, F., Uilenberg, G. and Fransen, F.F.J (1988). Antigenic differences between stocks of *Cowdria ruminantium. Research In Veterinary Science*, **44**:186-189.

Jongejan, F. and Thielemans M.J.C. (1989a). Identification of an immunodominant antigenically conserved 32 kilodalton protein antigen from *Cowdria ruminantium. Infection and Immunity,* **57**:3243-3246.

Jogejan F. and Thielemans M.J.C. (1989b). Antigenic diversity of Cowdria ruminantium isolates determined by cross-immunity.

Jongejan, F., Wassink L.A., Thielemans, M.J.C., Perie, N.M., and Uilenberg, G. (1989). Serotypes of *Cowdria ruminantium* and relationship with *Ehrlichia phagocytophila* determined by immunofluorescence. *Veterinary Microbiology,* **21**:31-40.

Jongejan, F. (1991). Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with *in vitro* attenuated Rickettsiae. *Infection and Immunity,* **59**:729-731.

Jongejan, F., Thielemans, M.J.C., De Groot, M., Van Kooten P.J.S. and van Zeijst, B.A.M. (1991a). Competetive enzyme linked immunosorbent assay for heartwater disease using monoclonal antibodies against *Cowdria ruminantium* specific 32 kilodalton protein. *Veterinary Microbiology,* **28**:119-121.

Jongejan, F., Thiemans, M.J.C., Barre, C. and Uilenberg G. (1991b). Antigenic diversity of *Cowdria ruminantium* isolates determined by cross-immunity. *Research in Veterinary Science,* **51**:24-28.

Jongejan, F., Zandbergen, T.A., van Wiel, P.A., De Groot, M. and Uilenberg G. (1991c). The tick-borne rickettsia *Cowdria ruminantium* has a *Chlamydia*-like life developmental cycle. *Onderstepoort Journal of Veterinary Research.* **58**:227-237.

Jongejan, F., De Vries, N., Nieuwenhuij, J., vanVliet, A.H.M. and Wassink, L.A. (1993a). The immunodominant 32 kilodalton protein of *Cowdria ruminatium* is conserved within the genus *Ehrlichia*, *Revue Elevue Medicine Veterinaire Pays Tropicaux*, **46**:145-152.

Jongejan, F., Vogel, S.W., Gueye,A. and Uilenberg, G. (1993b). Vaccination against heartwater using *in vitro* attenuated *Cowdria ruminantium* organisms. *Revue d'Elevage et de Medicine Veterinaire des Pays Tropicaux,* **46**:223-227.

Karrar, G. (1960). Rickettsial infection (heartwater) in sheep and goats in the Sudan. *British Veterinary Journal,* **116**:105-114.

Karrar, G. and Elhag Ali, B. (1965). Oral treatment of heartwater with oxytetracycline (Terramycine soluble powder). *British Veterinary Journal,* **121**:28-33.

Karrar, G. (1966). Further studies on the epizootiology of heartwater in the Sudan. *Sudan Journal Veterinary Science Animal Husbandry*, **6**: 83-85.

Kaufmann, S.H.E. (1988). Immunity against intracellular bacteria. Biological effector functions and antigen specificity of T lymphocytes. *Current Topics in Microbiology and Immunology*, **138**:141-169.

Kaufmann, S.H.E. (1993). Immunity to intracellular bacteria. *Annual Review of Immunology*, **11**: 129-163.

Kaufmann, S.H.E. (1995). Immunity to intracellular microbiol pathogens. *Immunology Today*, **16**:338-342.

Kaylor, P.S., Crawford, T.B., Mclewan, T.F. and Palmer, G.H. (1991). Passive transfer of antibody to *Ehrlichia risticii* protects mice from Ehrlichiosis. *Infection and Immunity*, **59**:2058-2062.

Kazar, J., Skultetyova, E. and Brezina, R. (1975). Phagocytosis of *Coxiella burnetti* by macrophages. *Acta Virologia Prague*, **19**:426-431.

Kazar, J., Schramek, S., and Brezina, R. (**1997**). Analysis of antibody response and immunoglobulins in sera of rabbits and guinea pigs immunised with Coxiella burnetti. *Acta Virologica*, **21**:246-255.

Kekcheeva, N.G., Vovk, O.A., Abrosimova G.E., Chereshkova E.A., (1981). Activity of macrophages from guinea pigs at various periods after infection with *Rickettsia prowazekii. Acta Virologica*, **25**:150-154.

Kemeny, D.M., Noble, A. Homes, B.J. and Diaz-Sanchez D. (1994). Immune regulation. a new role for the $CD8^+$ T cell. *Immunology Today*, **15**:107-110.

Kenisson, D.C., Elsasser, T.H. and Fayer, R. (1991). Tumor necrosis factor as a potential mediator of metabolic and hormonal responses to endotoxemia in calves. *American Journal of Veterinary Research*, **52**:1320-1326.

Kishimoto, R.A. and Walker J.S. (1976). Interaction between *Coxiella burnetti* and guinea pig peritoneal macrophages. *Infection and Immunity*, **24**:416-421.

Kobold, A. M., Martinez D, Camus E. and Jongejan, F. (1992). Distribution of heartwater in the Caribbean determined on the basis of detection of antibodies to the conserved 32 kilodalton protein. *Journal Clinical Microbiology*, **30**:1870-1873.

Kocan, K.M. and Bezuidenhout, J.D. (1987). Morphology and development of *C. ruminantium* in *Amblyomma* ticks. *Onderstepoort Journal of Veterinary Research*, **54**:177-182.

Kocan, K.M., Bezuidenhout, J.D. and Hart. A. (1987). Demonstration and ultrastructural features of *Cowdria ruminantium* in the midgut and salivary cells of nymphal *Amblyomma hebraeum. Onderstepoort Journal of Veterinary Research*, **54**:87-92.

Kocan, K.M.; Morzaria, S.P., Voigt, W.P, Kiarie, J. and Irvin, A.D. (1986). Demonstration of colonies of *Cowdria rumunantium* in the midgut of epithelial cells of *Ambylomma variegatum. American Journal of Veterinary Research*, **48**:356-360.

Koerner, T.J., Hamilton, T.A. and Adams, D.O. (1987). Suppressed expression of surface IA on macrophage by lipopolysaccharides. Evidence of regulation at the level of Messenger RNA. *Journal of Immunology,* **39**:239-243.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature*, **227**:680-686.

Lally, N.C., Nicol S., Paxton, E.A., Cary, C.M. and Sumption, K.J. (1995). The *Cowdria ruminantium* GroEL operon. *Microbiology*, **141**:2091-2100.

Lanzavecchia, A. (1996) Mechanisms of antigen uptake for presentation. *Current Opinion in Immunology*, **8**:348-354.

Lawrence, J.A., Malika, J. Whiteland, A.P., Kafuwa, P.T. and Jongejan, F. (1993). Use of serological response to evaluate heartwater vaccination of cattle. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **46**:211-215.

Lawrence, J.A., Malika, J, Whiteland, A.P. and Kafuwa, P.T. (1995). Reactions of heartwater vaccination in crossbred Zebu cattle. *Onderstepoort Journal of Veterinary Research*, **62**:19-29.

Lee, E.H. and Rikihisa, Y. (1996). Absence of tumor necrosis factor alpha, Interleukin-6, (IL-6) and granulocyte-macrophage stimulating factor expression but presence of IL-1β, IL-8, and IL-10 in human monocytes exposed to viable or killed *Ehrlichia chaffeensis*. *Infection and Immunity*, **64**:4211-4219.

Lewis, E.A. (1939). The ticks of East Africa II: Tick diseases and their control. *Empire Journal of Experimental Agriculture*, **7**:299-301.

Lewis, E.A. (1949). Heartwater. Annual Report (1947). Veterinary Department, Kenya, 51.

Lewis, G.E. Jr., Hill, S.L., Ristic, M. (1978). Effect of canine immune serum on the growth of *Ehrlichia canis* with non-immune macrophages. *American Journal of Veterinary Research*, **39**:71-76.

Li, H., Jerrels, T.R., Spitalny, G.L. Walker, D.H. (1987). Gamma interferon as a crucial host defense against *Rickettsia conorii in vivo*. *Infection and Immunity*, **55**:1252-1255.

Logan, L.L., Birnie, E.F., Endris R.G. and Mebus C.A. (1985). Immunologic aspects of protection against *Cowdria rumunantium* in Workshop on diseases by leukocytic rickettsiae of man and animals. Urbana-Champaign, Illnois,USA, Abstract No. 36.

Logan, L.L., Birnie, E.F. and Mebus, C.A. (1987a). Cross immunity between isolates of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **54**:345

Logan, L.L., Whyard T.C., Quintero, J.C. and Mebus, C.A. (1987b). The development of *Cowdria ruminantium* in neutrophils. *Onderstepoort Journal of Veterinary Research*, **54**:197-204.

Logan, L.L., Tembley, Y.S. and Miller, O.K. (1988). The importance (*Cowdria ruminantium*) in Mali. *Bulletin of Animal Health and Production in Africa*, **36**:89-92.

Louis Trichardt (1838) Cited by Provost A. and Bezuidenhout J.D. (1987). In The historical Background and Global Importance of Heartwater. *Onderstepoort Journal of Veterinary Research*, **54**:165-169

Lounsbury, C.P. (1900). Tick heartwater experiments. *Agricultural Journal Cape of Good Hope*, **16**: 682-687.

Lukacs, K., Roth, J.A. and Kaeberle, M.N. (1985). Activation of neutrophils by antigen induced lymphokine with emphasis on antibody-independent cytotoxicity. *Journal Leucocyte*, **38**:557.

MacDonald, G.A., Anacker, R.L., Garjian, K. (1987). Cloned gene of *Rickettsial rickettsii* surface antigen:candidate vaccine for Rocky Mountain spotted fever. *Science*, **235**:83-85.

Macdonald, G.A., Maclean, J.D. Mann, R.E. and Milch, L.J. (1988). Protection of guinea pigs from experimental Rocky Mountain spotted fever with a cloned antigen of *Rickettsia rickettsii. Journal of Infectious Diseases*, **158:**228-231.

Mackay C.R., Maddox, J.F. and Brandon, M.R. (1987). Lymphocyte antigens of sheep:identification and characterisation using a panel of monoclonal antibodies. *Veetrinary Immunology and Immunopathology*, **17**:91-102.

Mackay, C.R., Hein, W.R., Brown, M.H. and Matzinger, P. (1988). Unusual expression of CD2 in sheep. Implications for T cell interactions. *European Journal of Immunology*, **18**:1681-1886

Mackenzie, P.K.I and van Rooyen, R.E. (1981). The isolation and culture of *Cowdria ruminantium* in albino mice. *Proceedings of an International Conference on Tick Biology and Control, Rhodes University Grahamstown, South Africa*, 33-39.

Mackenzie, P.K.I. and McHardy, N. (1984). The culture of *Cowdria ruminantium* in mice: significance in respect of epidemiology and control of heartwater. *Preventive Veterinary Medicine*, **2** 227-237.

Mahan, S.M., Tebele, N., Mkwedeya, D., Semu, S., Nyathi, C. B., Wassink, L.A., Kelly, P.J.; Petre, T. and Barbett, A.F. (1993). An immunoblotting diagnostic assay for heartwater based on the immunodominant 32 kilodalton protein of *Cowdria ruminantium* detects false positives in field sera. *Journal of Clinical Microbiology*, **31**:2729-2737

Mahan, S.M., Andrew, H.R., Tebele, N., Burridge, M.J. and Barbet, A.F. (1994a). Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Research in Veterinary Science*, **58**:46-49.

Mahan, S.M., McQuire, T.C., Semu, S.M., Bowie, M.V., Jongejan, F., Rurangirwa, F.R. and Barbett, A.F. (1994b). Molecular cloning of a gene encoding the immunodominant 21 kilodalton protein antigen of *Cowdria ruminantium Microbiology,* Reading, **140**:2135-2142.

Mahan, S.M. (1995). Review of the molecular biology of *C. ruminantium. Veterinary Parasitology*, **57**:51-56.

Mahan, S.M., Andrew H.R., Tebele N., Burridge, M.J. and Barbett, A.F. (1995). Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium. Research in Veterinary Science*, **58**:46-49.

Mahan, S.M., Sileghem, M., Smith, G.E. and Byrom, B. (1996). Neutralisation of bovine Concanavalin A T cell supernatant-mediated anti-*Cowdria ruminantium* activity with antibodies specific to Interferon gamma but not tumor necrosis factor. *Parasite Immunology*, **18**: 317-324.

Manor, E. and Sarov, I. (1990). Tumor necrosis alpha and prostaglandin E2 production by human monocyte-derived macrophages infected with spotted fever group Rickettsiae. *Annals of New York Academy of Science*, **590**:157-167.

Martinez, D., Sheikboudou, C., Couraud, P.O and Bensaid, A. (1993). Protection of goats against heartwater is acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium. Revue Elevue Medicine Veterinaire Pays Tropicaux*, **46**:229.

Martinez, D.J.C., Maillard, S., Cisne, Sheikboudou, C. and Bensaid, A. (1994). Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *C. ruminantium. Veterinary Immunology Immunopathology*, **41**:153-163.

Mason, J.H. and Alexander, R.A. (1940). The susceptibility of ferret to heartwater. *Journal of South African Veterinary Medical Association*, **11**:98-107.

McHardy N and Mackenzie, P.K.I. (1984). Virulence of two strains of *Cowdria ruminantium* and their drug use to predict drug activity against heartwater. *Tropical Animal Health and Production* **16**:56-62.

McHardy, N. and Mackenzie P.K.I. (1987a). Identification of drugs effective against *Cowdria ruminantium* using a mouse screening system. *Onderstepoort Journal of Veterinary Research*, **54**:481-484.

McHardy, N. and Mackenzie, P.K.I (1987b). Virulence of two strains of *Cowdria ruminantium* in mice and their use to predict drug activity against heartwater. *Tropical Animal Health Production*, **16**:56-62.

McQuire, T.C., Musoke, A.J. and Kurth, T. (1979). Functional properties of bovine $IgG_1$ and $IgG_2$: interaction with complement, macrophages neutrophils and skin. *Immunology,* **38**:249-256.

Mebus, C.A. and Logan L.L. (1988). Heartwater disease of domestic and wild ruminants. *Journal of American Veterinary Medical Associaton*, **192**:950-952.

Miller, M.A., Skeen, M.J. and Ziegler, H.K. (1995). Non viable bacterial antigens administered with IL-12 generate antigen specific T cell responses and protective immunity against *Listeria monocytogenes*. *Journal of Immunology*, **155**-4817-4828.

Mohan, R. N. (1968). Diseases and parasites of Buffaloes Part I. Viral Mycoplasma and rickettsial diseases. *Veterinary Bulletin*, **38**:567-576.

Mombaerts, P., Arnoldi, J.;F., Russ and Tonegawa, S. (1993). Different roles of alpha beta ($\alpha\beta$) and gamma delta ($\gamma\delta$) T cells in immunity against intracellular pathogen. *Nature* (London), **365**:53-56.

Moshkovskii, S.D. (1947). Comments by readers. *Science*, **106**:62.

Mossman, T.R. and Coffman, R.L. (1989). TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annual Reviews Immunology*, **7**:145-73.

Mutunga, M. and Sumption K.J. (1995). Nitric oxide production in *Cowdria ruminantium* infected bovine endothelial cells *in vitro*: effect of interferon gamma on the nitric oxide production, the organism and cell viability. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **89**:592.

Nakane, A.T., Minagawa, M., Kohanawa, Y., Chen, H., Sato, M. Moryama, M. and Tsuruoka, N. (1989). Interaction between endogenous gamma interferon and tumor necrosis factor in host resistance against primary and secondary *Listeria monocytogenes* infection. *Infection and Immunity*, **127**:3331-3337.

Neitz, W.O. (1933) The blesbuck (*Damaliscus albifrons* ) as a carrier of heartwater and bluetongue. *Journal South African Veterinary Medical Association*, **4**:34-36.

Neitz, W.O. (1937). The transmission of heartwater by means of the bont tick (*Amblyomma hebraeum*). *Onderstepoort Journal of Veterinary Research*, **9**:37-46.

Neitz, W.O. (1939). The immunity of heartwater. *Onderstepoort Journal of Veterinary Research*, **13**: 245-283.

Neitz, W.O. (1940). Uleron in the treatment of heartwater. *Journal of South African. Veterinary Medical Association*, **11**:15.

Neitz, W.O. (1968). Heartwater. *Bulletin Office Internationale Epizootics*, **70**:329-336.

Neitz, W.O., Alexander, R.A. (1941). The immunisation of calves against heartwater. *Journal of South African Veterinary Medical Association*, **12**:103-111.

Neitz, W.O. and Alexander, R.A. (1945). Immunisation of cattle against heartwater and control of the tick-borne diseases, redwater gall sickness and heartwater. *Onderstepoort Journal of Veterinary Research*, **20**:137-152.

Neitz, W.O. Alexander, R.A. and Adelaar, T.F. (1947). Studies on immunity in heartwater. *Onderstepoort Journal of Veterinary Research*, **21**:243-253.

Neitz, W.O. (1968). Heartwater. Bulletin off Internationale. Epizootics. 70:329-336.

Neitz, A.W.H., Wiljoen, G.J., Bezuidenhout, J.D., Oberem, P.T., Visser, L. and Vermuelen, N.M.J. (1986). Detection of *Cowdria ruminantium* antigens during the course of heartwater disease by means of an enzyme linked immunosorbent assay. *Ondertepoort Journal of Veterinary Research*, **53**:205-207.

Ngumi, P.N.(1997). PhD Thesis characterisation of, and immunisation against heartwater (*Cowdria ruminantiun* infection) in sheep in Kenya University of Edinburgh.

Ngumi, P.N., Rumberia, M., Williamson, S.M., Sumption, K.J., Lesan, A.C. and Kariuki, D.P. (1997). Isolation of the causative agent of heartwater (*Cowdria ruminantium*) from three *Amblyomma* species in eight districts of Kenya. *Veterinary Record*, **140**:13-16.

Norval, R.A.I. (1978, 1979). Control of heartwater in lowveld cattle. *Rhodesian Research Index*, 1978, 106.

Norval, R.A.I. and Lawrence, J.A. (1979). The control of heartwater in Zimbabwe/Rhodesia. *Rhodesian Journal of Agricultural Research*, **76**:161-165.

Norval, R.A.I., Andrew, H.R. and Yunker, C.E (1989a). Pheromone mediation of host selection in bont ticks (*Amblyomma hebraeum* Koch). *Science* (Washington), **243**: 364-365.

Norval, R.A.I., Butler, J.F. and Yunker, C.E. (1989b). Use of carbon dioxide and natural or synthetic aggregation-attachment pheromone of the bont *tick Amblyomma hebraeum* to attract and trap unfed adults in the field. *Experimental and Applied Acarology*, **7**:171-180.

Norval, R.A.I. (1991). Heartwater disease. Developing World Agriculture. Grosvenor Press International Ltd., 180-185.

Norval, R.A.I., Yunker, C.E., Duncan, I.M. and Peter, T. (1991). Pheromone acaricide mixtures in the control of the tick *Amblyomma hebraeum. Experimental Acarology*, **11**:233-240.

Oberem, P.T. and Bezuidenhout J.D. (1987a). Production of heartwater vaccine. *Onderstepoort Journal of Veterinary Research*, **54**:481-484.

Oberem, P.T. and Bezuidenhout, J.D. (1987b). Heartwater in hosts other than domestic ruminants. *Onderstepoort Journl of Veterinary Research*, **54**:271-275.

Ojcious, D.M., Gachelin, G. and Dautry-Varsat, A. (1996). Presentation of antigens derived from microorganisms residing in host-cell vacoules. Trends in Microbiology **4**:53-59.

Okoh, A.E.J., Oyetunde.I.L. and Ibu, J.O (1987). Heartwater infection (cowdriosis) in a Sitatunga (*Tragelaphus spekii*). in Nigeria. *Journal of Wildlife Diseases*, **23**:211-214.

Owen, N.C., Littlejohn, A., Kruger, J.M. and Erasmus, B.J. (1973). Physiopathological features of heartwater in the sheep. *Journal of the South African Veterinary Medical Association,* **44**:397-403

Palmer, G.H., Kocan, Barron, S.J., Hair, J.A., Barbet, A.F., Davis and W.C. McQuire, T.C. (1986). Presence of common antigens including major surface protein epitopes between the catle (intraerythrocytic) and tick stages of *Anaplasma marginale*. *Infection and Immunity*, **50**:881-886.

Palmer, G.H., Orberle, S.M., Barbet, A.F., Davis, W.C., Goff, W.L. and Mcquire, T.C. (1988). Immunisation with a 36 kilodalton suface protein induces protection against homologous *Anaplasma marginale* challenge. *Infection and Immunity*, **56**:1526-1531.

Park, J. and Rikihisa, Y. (1991). Inhibition of *Ehrlichia risticii* in murine peritoneal macrophages by gamma interferon a calcium ionophore and Concanvalin A. *Infection and Immunity*, **59**:3418-3423.

Paxton, E.A., Sumption, K.J. and Pow, E. (1993). Western blot analysis of *Cowdria ruminantium* antigens and detection of cross-reactions with *Ehrlichia phagocytophila* and *E. ondiri* (ODA Report).

Pegram, R.G., Hoogstraal, H. and Wassef, H.Y. (1981). Ticks (*Acari:ixodoidea*) of Ethiopia. I. Distribution, ecology and host relationshipss of species infesting livestock. *Bulletin of Entomological Research,* **71**:339-359.

Pegram, R.G. and Chizyuka, H.G.B. (1990).The impact of natural infestation of ticks in Zambia on the productivity of cattle and its implications for control strategies in central Africa. In *Proc. FAO Expert Consultation on Revision of the Strategies for the Control of Ticks and Tick-Borne Diseases, Rome, 22-59 September 1989. Parastologia,* **32**:165-176.

Pelissier, A., Troquereau, P. and Triquier, E. (1950). Etudes sur les rickettsioses humaines et.anmales en Afrique Equatoriale Francaise.III Rickettsiose generale du boeuf difference de la heartwater. *Bulletin Societe Pathologie Exoticue*, **43**: 168-176 (cited by Camus *et al.,* 1996).

Perreau, P., Morel, P.C., Barre, N. and Durand, P. (1980). Existence de la cowdriose (heartwater) 'a *Cowdria ruminantium*, chez les petits rumints des Antilles Francaises (la Guadeloupe) et des Mascareignes (la Re'union et Ile Maurice). *Revue d'Elevage et de Medecine des Veterinaire Tropicaux*, **33**:1,21-22.

Pienaar, J.G. (1970). Electron microscopy of *Cowdria* (*Rickettsia*) *ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. *Onderstepoort Journal of Veterinary Research*, **37**:67-78.

Pienaar, J.G., Basson, P.A. and van der Merwe, J.W. De. B. (1966). Studies on the pathology of heartwater *Cowdria* (*Rickettsia*) *rumiantium* (Cowdry, 1926). 1. Neuropathological changes. *Onderstepoort Journal of Veterinary Research*, **33**:115-138.

Porter, P. (1979). Structural and functional characteristics of immunoglobulins of the common domestic species. *Advances in Veterinary Science and Comparative Medicine*, **23**:1-19.

Pow, E., Paxton E.A. and Sumption, K.J. (1993). Culture *in vitro* of *Cowdria ruminantium* in bovine endothelial cells. *Transactions Royal Society Tropical Medicine Hygiene*. 8723.

Provost, A. and Bezuidenhout, J.D. (1987). The historical background and global importance of heartwater. *Onderstepoort Journal of Veterinary Research*, **54**:165-169.

Prozesky, L. and Du Plessis, J.L (1985a). The pathology of heartwater. I: A study of mice infected with the Welgevonden strain of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **52**:71-79.

Prozesky, L. and Du Plessis, J.L. (1985b). The pathology of heartwater. II: A study of the lesions in sheep and goats infected with the Ball 3 strain of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **52**:81-85.

Prozesky, L. and Du Plessis, J.L. (1985c). Heartwater in Angora goats II. A pathological study of artificially infected treated and untreated goats. *Onderstepoort Journal of Veterinary Research,* **52**:13-19.

Prozesky, L., Bezuidenhout, J.D. and Patterson C.L. (1986). Heartwater: an *in vitro* study of the ultrastructure of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **53**:153-159.

Prozesky, L. (1987a). Heartwater :The morphology of *Cowdria ruminantium* and its staining characteristics in the vertebrate and *in vitro*. *Onderstepoort Journal of Veterinary Research*, **54**:173-176.

Prozesky, L. (1987b).The pathology of heartwater III A Review. *Onderstepoort Journal of Veterinary Research*, **54**:281-286.

Prozesky, L. and Du Plessis, J.L. (1987). Heartwater: The development and life cycle of *Cowdria ruminantium* in the vertebrate host, ticks and cultured endothelial cells. *Onderstepoort Journal of Veterinary Research*, **54**:193-196.

Purchase, H.S. (1945). A simple and rapid method demonstrating *Rickettsia rumunantium* (Cowdry, 1926) in heartwater brains. *Veterinary Record*, **57**:413-414.

Purnell R.E. (1984). Control of heartwater in Southern Africa with Terramycin /LA. *Preventive Veterinary Medicine*, **2**:239-254.

Ramise, J. and Uilenberg, G. (1971). Etude sur la cowdriose a Madagascar. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **24**:519-522 (cited by Uilenberg 1983).

Redl, H., Schlag, G. and Lamche, H. (1990). TNF-and LPS induced changes in the lung vascular permeability studies in un-anethesthetised sheep. *Circulatory Shock*, **31**:183-192.

Reiner, N.E. (1987). Parasite accessory cell interactions in murine Leishmaniaisis. Evasion and stimulus dependent suppression of macrophage Interleukin 1 responses by *Leishmania donovani*. *Journal of Immunology*, **138**:1919-1925.

Rossouw, M., Neitz, A.W.H., De Waal, D.T., Du Plessis, J.L., Van Gas, L. and Brett, S. (1990). Identication of antigenic proteins of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, **57**:215-221.

Roth, J.A. (1994). Neutrophils and killer cells *In Cell Mediated Immunity in Ruminants,* pp. 127-136 (Eds. Bruno Goddeeris, and Ivan Morrison), CRC, Press Inc.

Schindler, R, Mancilla, J., Endres S., Ghorbani R., Clark, S.C. and Dinarello C.A. (1990). Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human mononuclear cells:Il-6 suppresses IL-1 and TNF *Blood,* **75**:40-47.

Schirmbeck, R., Melber, K., Mertens, T. and Reimann, J. (1994). Selective stimulation of murine cytotoxic T cell and antibody responses by particulate or monomeric hepatits B virus surface (S) antigen. *European Journal of Immunology*, **24**:1088-1095.

Schmeer, N., Kraus, H., Lochbarcht, W. and Weigand, D. (1986). Differences in $IgG_1$ and $IgG_2$ responses of cattle infected with *Coxiella burnetti* and following vaccination. *Comparative Immunology and Microbiology Infectious Diseases*, **9**:95-98.

Schmeer, N., Kraus, H., Apel, A.W.P, Perz-Martinez, J.A. and Reiser, H. (1987). Analysis of caprine $IgG_1$ and $IgG_2$ subclass responses to *Chlamydia psittaci* infection and vaccination. *Veterinary Microbiology,* **14**:125-135.

Schmeer, N., Schnorr, K.L., Perez-Martinez J.A and Storz, J. (1987). Dominance of *Chlamydia psittaci* specific $IgG_2$ subclass in the humoral response of naturally and experimentally infected cattle. *Veterinary Immunology and Immunopathology*, **15**:311-322.

Scott, G.R. (1987). The taxonomic position of the causative agent of heartwater. *Onderstepoort Journal of Veterinary Research*, **54**:257-260.

Semu, S.M., Mahan, S. M., Yunker, C.E. and Burridge, M. J. (1992). Development and persistence of *Cowdria ruminantium* antibodies following experimental infection as detected by the indirect flourescent antibody test. *Veterinary Immunology and Immunopathology*, **33**:339-352.

Shankarapa, P.B., Dutta, S. and Mattingly-Napier, B. (1991). Identification of the protective 44 kilodalton recombinant Antigen of *Ehrlichia risticii. Infection and Immunity*, **60**:612-617.

Shemer, Y. and Sarov, I. (1988). Interferon and nonviral pathogens. In G.I Byrne and Turco J (Editors). Immunology Series Vol. 42. Marcel Dekker pp. 73-94.

Shenker, B.J., Berthold P. Dougherty P. and Porter K.K (1987). The immunosuppressive effects of *Centipeda periodontis*: selective cytotoxicity for lymphocytes and monocytes. *Infection and Immunity,* **55**:2332-2340.

Shenker B.J. and Slots J. (1989). Immunomodulatory effects of *Bacteriodes* products on in vitro human lymphocyte functions. *Oral Microbiology and Immunology*. **4**:24-29.

Shenker, B.J. Vitale, L. and Slots J. (1991). Immunosppressive effects of *Provetella intermedia* on *in vitro* human lymphocyte activation. *Infection and Immunity*, **59**:4583-4589.

Shirai, A., Catanzaro, P.J., Philiphs S.M. and Osterman J.V. (1976). Host defences in experimental scrub typhus: role of cellular immunity in heterologous protection. *Infection and Immunity*, **14**:39-46.

Shizuo, A., Tetsuya, T. and Tadamitsu, K. (1993). Interleukin-6 in Biology and Medicine. *Advances in Immunology*, **54**:1-52.

Silva, C.L and Lowrie, D.B. (1994). A single mycobacterial protein (Hsp65) expressed by a transgenic antigen-presenting cell vaccinates mice against tubercullosis. *Immunology*, **82**:244-248.

Silva, C.L., Silva, M.F., Pietro, R.C.L.R. and Lowrie D.B. (1994). Protection against tubercullosis by passive transfer with clones recognising mycobaterial heatshock protein 65. *Immunology*, **83**:341-346.

Soldan, A.W., Norman T.L., Masaka, S., Paxton E.A., Edelsten, R.M. and Sumption K.J. (1993). Seroconversion to *Cowdria ruminantium* of Malawi zebu calves reared under different tick control strategies. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **46**:171-177.

Splitter, G.A. and Everlith, K.M., (1989). *Brucella abortus* regulates bovine macrophage T cell interleukin-1 interaction by Major-Histocompatibility complex and interleukin-1 expression. *Infection and Immunity,* **57**:1151-1157.

Spruell, J. (1904). Heartwater inoculation experiments. *Agriculture Journal of Cape of Good Hope,* **24**:433-442. (Cited by Provost and Bezuidenhout 1987).

Spreull J. (1922). Heartwater. *Journal of the Department of Agriculture, Union of South Africa*, **4**:236-245 (Cited by Provost and Bezuidenhout 1987).

Stampa, S. (1969). Experiences with heartwater eradication. *Proceedings Symposium Biology and Control of Ticks in Southern Africa*, Grahamstown, 133-150.

Steck, W. (1928). Pathological studies on heartwater. 13th and 14th Report ofthe Director of Veterinary Research. Union of South Africa, 283-305. Cited by Prozesky (1987b).

Steinberg, M.J. Webb, S.A. and Roth, J.A. (1989). Role of arachidonic acd metabolism and protein synthesis in recombinant interferon induced activation of bovine neutrophils. *Leukocyte Biology*, **46**:450.

Stewart, C.G. (1987). Specific immunity in farm animals to heartwater. *Onderstepoort Journal of Veterinary Research*, **54**:341-342.

Storz, J. and Kaltenboeck, B. (1993) The Chlamydiales. *In Rickettsial and Chlamydial Diseases of Domestic Animals* (Editors Woldehiwet Z. and Ristic Miodrag), Pergamon Press, Oxford, New York, Seoul, Tokyo, pp. 27-64.

Stover, C.K., Marana, D.P., Dasch, G.A. and Oaks, E.V. (1990). Molecular cloning and sequence analysis of the Sta58 Major antigen gene of *Rickettsia tsutsugamushi*: Sequence homology and antigenic comparison of Sta58 to the 60 kilodalton Family of stress proteins. *Infection and Immunity*, **58**:1360-1368.

Synge, B.A. and Scott, G.R. (1976). Treatment of heartwater. Annual Report 1975-1976, Centre for Tropical Veterinary Medicine Edinburgh, 20.

Tafesse, B. (1992). MSc studies on the immunisation of goats and mice against heartwater with inactivated preparations of *C. ruminantium* (Cowdry 1925), University of Edinburgh.

Theiler, A. (1904, 1905, 1906). Immunisation against heartwater. Transvaal Annual Report to the Director of Agriculture, 121-122 cited by Bezuidenhout *et al.*, (1994)

Theiler, A. and Du Toit, P.J. (1928). The transmission of tick-borne diseases by the intrajugular injection of the emulsified intermediary host itself. *Bulletin de la Societe de Pathologie Exotique*, **19**:8, 725-737 (cited by Uilenberg 1983).

Thomas, A.D. (1957). Heartwater can be controlled. *Farming in South Africa*, 33:19.

Thomas, A.D. and Mansvevelt, P.R. (1957). Immunisation of goats against heartwater. *Journal of South African Veterinary Medical Association*, **28**:163-168.

Tizard, I.R. (1992) Macrophages and antigen processing. In *Veterinary Immunology Introduction (*Ed. Tizard, I.), 4th Ed, 27-40. W.B Saunders Company Philadelphia.

Tizard, I.R. (1994). Interferons In *Cytokines in Animal Health and Disease* (Editors Michael J. Myers and Michael P. Murtaugh), Marcel Dekker Inc., 1995, pp 1-57.

Totte, P., Blankaert, D., Zilimwabagabo, P. and Werrenne, J. (1993). Inhibition of *Cowdria ruminanntium* yield by interferon alpha and gamma in endothelial cells. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **46**:189-194.

Totte, P., Jongejan, F. de Gee, A.L.W and Werrenne, J. (1994). Production of interferon in *Cowdria ruminantium* infected-cattle its effects on infected endothelial cell cultures. *Infection and Immunity*, **62**:2600-2604.

Totte, P., Vachiery, N., Martinez, D., Trap, I., Ballingall, K.T., MacHugh, N.D., Bensaid, A. and Werenne, J. (1966). Recombinant bovine interferon gamma inhibits the growth of *Cowdria ruminantium* but fails to induce MHC II following infection of endothelial cells. *Veterinary Immunology and Immunopathology*, **53**:61-71.

Totte, P., McKeever, D., Martinez, D. and Bensaid, A. (1997). Analysis of T cell responses in cattle immunised against heartwater by vaccination with inactivated bodies of *Cowdria ruminantium*. *Infection and Immunity*, **65**:236-241

Towbin, H., Staehelin, T. and Gordon, J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of National Academy Science, USA,* **76**:4350-4354.

Tringali, G.R., Montenegro, M.R., Walker, D.H. and Mansueto, S. (1983). Immunity and rickettsial infections. A Review. *Acta Tropica* ,**40**:311-320.

Turco, J. and Winkler, H.H. (1983a). Inhibition of the growth of *Rickettsia prowazekii* in cultured fibroblasts by lymphokines. *Journal of Experimental Medicine*, **157:**974-986.

Turco, J. and Winkler, H.H. (1983b). Cloned mouse interferon-gamma inhibits the growth of *Rickettsia prowazekii* in cultured mouse fbroblasts. *Journal of Experimental Medicine* **158**:2159:1264.

Turco, J. and Winkler, H.H. (1988). Interactions between *Rickettsia prowazekii* and cultured host cells : alterations induced by gamma interferon. In G.I.Byrne and J.Turco.ed., Interferon and Non-viral Pathogens pp.95-115, Marcel Dekker Inc., New York.

Turco, J. and Winkler, H.H. (1993). Role of nitric acid synthetase pathway in the inhibition of growth of interferon sentive and interferon resistant *Rickettsia prowazekii* strains in L929 cells treated with tumor necrosis factor alpha and gamma interferon. *Infection and Immunity,* **61**:4317-4325.

Uilenberg, G. (1971). Etude sur la Cowdriose a Madagascar. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **24**:239-249.

Uilenberg, G. (1977). Heartwater: Summary of background, present state of knowledge, future. With a note on other tick-borne rickettsial infections of ruminants. Second F.A.O. consultation on research on tick-borne diseases and their vectors, Rome, F.A.O. 13

Uilenberg, G.(1981). Heartwater disease In *Diseases of Cattle in the Tropics*. The Hague, Boston, London, Martinus Nijhoff Publishers.

Uilenberg, G. (1982a). Experimental transmission of *Cowdria ruminantium* by the Gold Coast tick *Amblyomma maculatum*: danger of introducing heartwater and benign African theileriosis onto the American mainland. *Annual Veterinary Journal*, **43**:1279-1282

Uilenberg, G. (1982b). Disease problems associated with the introduction of European cattle in the tropics *Proceedings of the 12th World Congress on Diseases of Cattle* 7-10 September 1982, Amsterdam, The Netherlands.

Uilenberg, G., Corten, J.J.F.M. and Dwinger, R.H. (1982). Heartwater (*Cowdria ruminantium*) infection. *Sao Tome Veterinary Quarterly,* **4:**106-107.

Uilenberg, G. (1983). Heartwater (*Cowdria ruminantium* infection). Current Status. *Advances in Veterinary Science Comparative Medicine,* **27**:427-480.

Uilenberg, G., Zivkovic, D., Dwinger, R.H., Ter Huurne, A.A. and Perie, N.M. (1983). Cross-immunity between strains of *Cowdria ruminantium. Research in Veterinary Science,* **35**:200-205.

Uilenberg, G., Barre, N., Camus, E., Burridge, M.J. and Garris, G.I. (1984). Heartwater in the Caribbean. *Preventive Veterinary Medicine*, **2**:255-267.

Uilenberg, G., Camus, E. and Barre, N. (1985). Observations on a *Cowdria ruminantium* strain isolated from the Guadeloupe (French West Indies). *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **38**:34-42.

Uilenberg, G. (1989). Methods currently used for the control of heartwater ,their validity and proposal for future control strategies. *Proceedings of the FAO Expert Consultation on Revision of Strategies for the Control of Ticks and Tick-borne diseases,* Rome, 25-29 September 1989 (Griffiths R.B. and McCosker, P.J.I. Eds), *Parastologia Roma* 1990, **32**: 55-62.

Uilenberg, G. and Camus, E. (1993). Heartwater (Cowdriosis). *In Rickettsial and Chlamydial Diseases of Domestic Animals* (Woldehiwet Z and Ristic M. Eds) Pergamon Press, Oxford New York, Seoul, Tokyo, 293-332pp.

Van Amstel, S.R. and Oberem, P.T. (1987). Treatment of heartwater. *Onderstepoort Journal of Veterinary Research*, **54**:475-479.

Van Amstel, S.R., Oberem P.T., Didomencio, M., Kirkpatrick R.D. and Mathee J. (1988a). The presence of endotoxin activity in cases of experimental-induced heartwater in sheep. *Onderstepoort Journal of Veterinary Research*, **55**:217-220.

Van Amstel, S.R., Myburgh, E., Mundel, J. and Davidson, B. (1994). Studies on the presence of endotoxin activity and inflammatory products (Leukotrienes) in cases of experimentally induce heartwater in sheep. *Onderstepoort Journal of Veterinary Research*, **61**:29-33.

Van de Merwe, L. (1979). Field experience with heartwater (*Cowdria ruminantium*) in cattle. *Journal of the South African Veterinary Medical Association,* **50**: 323-325.

Van der Merwe, L. (1987). The infection and treatment method of vaccination against heartwater. *Onderstepoort Journal of Veterinary Research*, **54**:489-491.

Van Kleef, M., Neitz, A.W. H. and De Waal, D.T. (1992). Characterization of 27 and 31kDa proteins of *Cowdria ruminantium. Journal of the South African Veterinary Medical Association*, **59**:337-338.

Van Kleef, M., Neitz, A.W.H. and De Waal, D.T. (1993). Isolation of antigenic proteins of *Cowdria ruminantium. Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **46**:157-164.

Van Miert, A.S., van Duin, C.T. and Wensing, T. (1992). Fever and acute phase response induced in dwarf goats by endotoxin and bovine and human recombinant tumor necrosis factor. *Journal of Veterinary Pharmacology and Therapeutics*, **15**:335-342.

Van Pypekamp, H. and Prozesky, L. (1987). Heartwater an overview of the clinical signs, susceptibility and diferential diagnosis of the disease in domestic ruminants. *Ondersterpoort Journal of Veterinary Research,* **54**:263-266.

Van Saceghem, R. (1918). La peste du cheval ou horse sickness au Congo Belge. *Bulletin de. Societe Pathologue Exotique*, **11**:423-432. (cited by Camus and Barre, 1988)

Van Vliet, A., Jongejan, F. and Vander Zeijst, B. (1992). Phlogenetic position of *Cowdria ruminantium* based on analysis of amplified 16S rDNA sequences. *International Journal of Systemic Bacteriology*, **42**:494-498.

Van Vliet, A.N.M., Jongejan, F., van Kleef, M. and Vander Zeisjst, (1994). Molecular cloning, sequence analysis and expression of the gene encoding the immunodominant 32 kilodalton protein of *Cowdria ruminantium. Infection and Immunity*, **62**:1451-1456.

Van Vliet, A.H.M., Vander Zeisjst, B.A.M., Camus, E., Martinez, D. and Jongejan, F. (1995). The use of immunogenic region of the *Cowdria ruminantium* major antigenic protein in serological assay. *Journal of Clinical Microbiology,* **33**:2405-2410.

Van Winkelhoft, A.J. and Uilenberg, G. (1981).Heartwater: Cross immunity studies with strains of *Cowdria ruminantium* isolated in West and South Africa. *Tropical Animal Health and Production*, **13**:160-164.

Viljoen, G., Vermeulen, N.M.J. and Neitz, A.W.H. (1987). Theoretical aspects of the enzyme linked immunosorbent assay. Technique and its use in the detection of *Cowdria ruminantium* antigen and antibody in reacting animals. *Onderstepoort Journal of Veterinary Research*, **54**:305-312.

Viswanath, S., McDonald, G.A. and Watkins, N.G. (1990). Recombinant *Rickettsia conorii* vaccine protects guinea pigs from experimental Boutounneuese fever and Rocky Mountain spotted fever. *Infection and Immunity*, **58**:646-653.

Walker, J.B. and Olwage A. (1987). The tick vectors of *Cowdria ruminantium* (*Ixodoidea:Ixodedea* genus *Amblyomma*) and their distribution. *Onderstepoort Journal of Veterinary Research*, **54**:353-379.

Watson, D.L. (1988). Vaccination against experimental Staphylococcal mastitis in ewes. *Research in Veterinary Science*, **45**:16-21.

Weiss, K.E., Haig, D.A. and Alexander, R.A. (1952). Aureomycin in the treatment of heartwater. *Onderstepoort Journal of Veterinary Research*, **25**:41.

Wisseman C.L. Jr and Waddel A. (1983). Interferon -like factors from antigen- and mitogen-stimulated human leukocytes with anti-rickettsial and cytolytic action on *Rickettsia prowazekii.* Infected human endothelial cells, fibroblasts and macrophages. *Journal of Experimental Medicine* **157**:1780-1793.

Woldehiwet Z. and Sharma, R. (1990) Alterations in lymphocyte subpopulations in peripheral blood of sheep persistently infected with Border Disease virus. *Veterinary Microbiology*, **22**:153-160.

Woldehiwet, Z. and Ristic, M. (1993). The rickettisae in Rickettsial and Chlamydial diseases of domestic animals (Woldehiwet, Z and Ristic M. Eds). Pergamon Press, Oxford, New York, Seoul, Tokyo, 1-26 pp.

Woldehiwet, Z. and Scott, G.R. (1993). Tick-borne (pasture) fever in Rickettsial and Chlamydial diseases of domestic animals (Woldehiwet, Z and Ristic M. Eds). Pergamon Press, Oxford, New York, Seoul, Tokyo, 233-254 pp.

Wyrick P.B., Brownridge E.A and Ivins B.E (1978).Interaction of *Chlamydia psittaci* with mouse peritoneal macrophages. *Infection and Immunity* **19**:1061-1067.

Young, A.S., Groocock, C.M. and Kariuki, D.P. (1987). Intergrated control of ticks and tick-borne diseases of cattle in Africa. *Parasitology*, **96**: 403-432.

Young, D. Lathigra, R., Hendrix, R., Sweetser, D. and Young, R. A. (1988). Stress proteins are immune targets in leprosy and tuberculosis. *Proceedings of National Academy of Science(* USA) 85:426-4270.

Yunker C.E.,Byrom, B. and Semu S. (1988). Cultivation of Cowdria ruminiantium in bovine vascular endothelial cells, *Kenya Veterinarian*, **12**:12-16.

# APPENDICES

**Appendix A.1**
Growth Medium for Heartwater Cultures

| To make 100ml of medium | |
|---|---|
| Glasgow MEM (BHK-21) | 100 ml |
| Tryptose phosphate broth | 10 ml |
| Foetal bovine serum | 10 ml |
| 2 mM L-glutamine | 1 ml |
| 20 mM HEPES buffer | 2 ml |
| Antibiotic/Antimycotic | |
| penicillin sodium | 10,000 units |
| streptomycin | 10,000 µg |
| amphotericin B | 25 µg/ml |

**Appendix A.2**
Reagents and Buffers For Indirect ELISA for the Detection of $IgG_1$ and $IgG_2$ Isotype Antibody Responses.

ELISA coating buffer
Carbonate/bicarbonate buffer pH 9.0 (Sigma)
Dissolve one capsule in 100 ml deionised water to give 0.05M buffer pH 9.6 at 25°C.

| Washing buffer | |
|---|---|
| 0.9% NaCl-Tween 20 | |
| NaCl | 9 gm |
| Tween-20 | 0.5 gm |
| Distilled water(MillQ) | 1 litre |

**Appendix A.3**

| PBS-Tween-20 (PBS, 0.05%T) | |
|---|---|
| PBS | 1 litre |
| Tween-20 | 0.5 gm |

**Appendix A.4**

| Blocking buffer (4% Normal rabbit serum/PBS/0.05% Tween) | |
|---|---|
| PBS-Tween-20 | 100 ml |
| Normal rabbit serum | 4.0 ml |

**Appendix A.5**

Buffers for purification of 35kDa subclone recombinant (Histag) protein of *C. ruminantium.*

Denaturing conditions.

Lysis buffers

Buffer B (pH 8.0) 8 M urea, 0.1 M $NaHPO_4$, 0.01 M Tris-HCl, pH 8.0.

Wash buffer

buffer C (pH 6.3) 8 M urea, 0.1 M $NaHPO_4$, 0.01 M Tris-HCl pH 6.3
32 mM Imidazole, 0.005% Triton® 100-X.

Elution buffers 8 M urea, 0.1 M $NaHPO_4$, 0.01 M Tris-HCl pH 4.5.

**Appendix A.6**

Reagents and gel preparation for SDS-PAGE slab gels (Laemmli buffer system)

Stock solutions

A. Acrylamide/bis (40%)

B. 1.5 M Tris -HCl, pH 8.8
27.23 g Tris base (18.15 g/100 ml)
~80 ml distilled water

Adjust pH to 8.8 with 1 N HCl. to make 150 ml with distilled water and store at 4°C

C. 0.5 M Tris-HCl, pH 6.8
6 g Tris base
~60 ml distilled water.

Adjust to pH 6.8 with 1 N HCl. Make to 100 ml with distilled water store at 4°C.

D. 10 % SDS
Dissolve 10 g SDS in water with gentle stirring and bring to 100 ml with $H_2O$.

Preparation of separating gels

| | 10 % | 12 % |
|---|---|---|
| Distilled water | 4.05 ml | 3.35 ml |
| 1.5M Tris-HCl pH 8.8 | 2.50 ml | 2.50 ml |
| 10% SDS | 100 µl | 100 µl |
| 40% Acrylamide | 3.0 ml | 2.50 ml |
| TEMED | 5 µl (0.5 µl/ml) | 5 µl |
| 10% Ammonium persulphate | 50 µl (5 µl / ml) | 50 µl |

| Stacking gel | |
|---|---|
| Distilled water | 6.10 ml |
| 0.5M Tris-HCl pH 6.8 | 2.50 ml |
| 10% SDS (w/v) | 1.0 ml |
| 40% Acrylamide | 1.0 ml |
| TEMED | 10 ml |
| 10% Ammonium persulphate | 50 µl (5 µl/ml) |

Sample buffer (SDS reducing buffer) stored at room temperature

| | Volume |
|---|---|
| Distilled water | 2.0 ml |
| 0.5M Tris-HCl pH 6.8 | 1.0 ml |
| Glycerol | 0.8 ml |
| 10% (w/v) SDS | 1.6 ml |
| 2-b-mercaptoethanol | 0.4 ml |
| 0.05 % (w/v) bromophenol blue | 0.2 ml |

Sample diluted at 1:1 with buffer and heated at 95°C for 5 minutes.

| 5X Electrode buffer (running) Buffer, pH 8.3 | |
|---|---|
| Tris base | 15 g/l |
| Glycine | 72 g/l |
| SDS | 5 g/l |

make up to 1000ml with distilled water
Store at 4°C. warm to 37°C before use if precipitation occurs

| Bjerrum and Schaefer-Nielsen transfer buffer | |
|---|---|
| 48 mM Tris | 5.82 g/l |
| 39 mM Glycine | 2.93 g |
| 1.3 mM SDS (0.0375) | 3.75 ml (10%) |
| 20% methanol | 200 ml |

Dissolve in dd$H_2O$ adjust volume to a litre

| Tris-buffered saline (TBS) | |
|---|---|
| 20 mM Tris | 24.22 g |
| 150 mM NaCl | 87.66 g |

Dissolve in de-ionised distilled water adjust the pH to 7.5 with 1 N HCl and make it up to 1 litre

**Appendix A.7**

Reagents for Coommassie Blue Staining and Destaining

10 % Acetic acid

40 % Methanol

50 % distilled water.

800 ml Methanol + 200 ml Acetic acid +1000 ml distilled water

**Appendix A.8**

Protein elution buffer

| | |
|---|---|
| 25 mM Tris base | 3.0 g |
| 192 mM Glycine | 14.4 g |
| 0.1 % SDS | 1.0 g |

to 1 litre with $dH_2O$

Store at 4°C. Warm to 37°C before use if precipitation occurs

Protein dialysis buffer

| | |
|---|---|
| 25mM Tris base | 3.0 g |
| 192 mM Glycine | 14.4 g |
| 0.01%SDS | 0.1 g |

to 1 litre with $dH_2O$

## APPENDIX B

**Appendx B Table 1.** Proliferative responses of PBMC from two IEB immunised goats (G107, G108) and control goat (G109) stimulated *in vitro* with EBs of the Ball 3 Gardel (heterologous) and Welgevonden (homologous) stocks of *C. ruminantium*. The responses were determined by MTS assay.

| Goat No. | Antigen concentration µg/ml | *C.ruminantium* stocks & OD values | | |
|---|---|---|---|---|
| | | Ball 3 | Gardel | Welgevonden |
| G107 IEB | 1 | 0.0435 | 0.0405 | 0.1033 |
| | 5 | 0.0485 | 0.043 | 0.089 |
| | 10 | 0.124 | 0.096 | 0.0615 |
| | 15 | 0.115 | 0.083 | -0.006 |
| | 20 | 0.1905 | 0.1068 | -0.013 |
| G108 IEB | | | | |
| | 1 | 0.143 | 0.096 | 0.169 |
| | 5 | 0.151 | 0.192 | 0.127 |
| | 10 | 0.162 | 0.167 | 0.1645 |
| | 15 | 0.205 | 0.168 | 0.144 |
| | 20 | 0.221 | 0.161 | -0.206 |
| G109 C | | | | |
| | 1 | -0.024 | -0.011 | -0.020 |
| | 5 | -0.020 | 0.007 | 0.018 |
| | 10 | -0.015 | 0.019 | 0.012 |
| | 15 | 0.002 | 0.043 | -0.032 |
| | 20 | 0.018 | 0.059 | -0.029 |

**Key:**
OD values are the medians of 3 tests taken at 570nm using a plate reader

**Appendix B Table 2.** T-cell counts ($10^9$) of immunised goats before and after challenge with virulent homologous culture of Welgevonden stock of *C. ruminantium.*

| group/goats | | No days post challenge | | | | | | |
|---|---|---|---|---|---|---|---|---|
| I/T | T cells | 0 | 3 | 7 | 10 | 17 | 21 | 32 |
| G74 | $CD4^+$ | 0.78 | 1.10 4 | 0.81 1 | 1.18 3 | 0.8 | 0.98 4 | 1.03 |
| | $CD8^+$ | 0.87 5 | 0.63 2 | 0.6 | 1.01 | 3.68 | 2.66 | 2.8 |
| | γδ | 0.15 1 | 0.10 4 | 0.04 | 0.11 7 | 0.13 2 | 0.15 3 | 0.16 |
| IEB gp | | | | | | | | |
| G107 | $CD4^+$ | 1.00 | 1.67 | 0.59 | 0.40 | | | |
| | $CD8^+$ | 0.77 | 1.49 | 0.3 | 0.59 | | | |
| | γδ | 0.17 | 0.24 | 0.06 | 0.13 | | | |
| G108 | $CD4^+$ | 5.17 | 4.00 | 3.46 | 1.29 | | | |
| | $CD8^+$ | 2.31 | 1.80 | 0.74 | 0.87 | | | |
| | γδ | 1.25 | 1.00 | 0.32 | 0.37 | | | |
| controls | | | | | | | | |
| G109 | $CD8^+$ | 2.97 | 0.94 | 0.89 | 0.79 | | | |
| | $CD4^+$ | 1.72 | 1.17 | 0.73 | 0.91 | | | |
| | γδ | 1.21 | 0.67 | 0.42 | 0.45 | | | |
| G110 | $CD8^+$ | 2.95 | 2.29 | 2.10 | 0.84 | | | |
| | $CD4^+$ | 2.63 | 1.12 | 2.28 | 4.48 | | | |
| | γδ | 1.23 | 1.14 | 0.68 | 0.45 | | | |

**Appendix B Table 3** Changes in $CD4^+$:$CD8^+$ ratios of goats after challenge with virulent culture of *C. ruminantium* (Welgevonden).

| | days post challenge & $CD4^+$:$CD8^+$ ratios | | | | | |
|---|---|---|---|---|---|---|
| Goat No. | 3 | 7 | 10 | 17 | 21 | 32 |
| G74 I/T | 2:1 | 1.3:1 | 1:1 | 1:5 | 1:2 | 1:2 |
| G107 IEB | 1:1 | 2:1 | 1:1 | | | |
| G108 IEB | 2:1 | 5:1 | 2:1 | | | |
| G109 C | 1:1 | 1:1 | 1:1 | | | |
| G110 C | 2:1 | 1:1 | 1:5 | | | |

**Key:**
I/T = infection /treatment, IEB = inactivated elementary bodies C = control.

**Appendix B. Table 4.** Counts ($10^9$) of PBMC of immunised and control goats before and after challenge with virulent stock of *C. ruminantium* (Welgevonden).

| Goat No/. group | cell type | days | post | challenge & counts | |
|---|---|---|---|---|---|
| | | 0 | 3 | 7 | 10 |
| I/T G74. | WBC | 6.30 | 10.80 | 7.40 | 10.80 |
| | Monocytes | 0.19 | 0.12 | 0.37 | 0.32 |
| | Neutrophils | 2.46 | 7.78 | 4.44 | 7.02 |
| IEB G107. | WBC | 11.50 | 14.70 | 16.20 | 13.20 |
| | Monocytes | 0.12 | 1.03 | 0.16 | 1.45 |
| | Nuetrophils | 6.56 | 6.76 | 12.78 | 8.10 |
| IEB G108. | WBC | 19.40 | 19.20 | 19.10 | 20.40 |
| | Monocytes | 0.190 | 0.19 | 0.57 | 2.24 |
| | Neutrophils | 4.66 | 6.53 | 6.11 | 9.20 |
| Control G109. | WBC | 19.30 | 12.60 | 12.10 | 15.50 |
| | Monocytes | 0.77 | 0.38 | 1.21 | 1.86 |
| | Neutrophils | 7.91 | 7.81 | 6.41 | 10.39 |
| Control G110. | WBC | 19.40 | 17.80 | 13.00 | 21.50 |
| | Monocytes | 0.58 | 0.53 | 0.91 | 2.58 |
| | Neutrophils | 6.79 | 9.43 | 3.12 | 10.32 |

**Key:**
I/T = infection /treatment, IEB = inactivated elementary bodies C = control.

**Appendix B Table 5.** Summary statistics for T cell counts ($10^9$/l)in peripheral blood of goats inoculated with live EB's (I/T), immunised with inactivated EB's (IEB) and controls following challenge with *C. ruminantium*. (Welgevonden).

a) $CD4^+$ counts.

| Goat No. | mean | median | stdev | SE mean |
|---|---|---|---|---|
| G74 I/T | 0.969 | 0.957 | 0.204 | 0.102 |
| G107 IEB | 0.914 | 0.793 | 0.563 | 0.281 |
| G108 IEB | 3.473 | 3.73 | 1.623 | 0.812 |
| G109 Control | 1.379 | 0.918 | 1.048 | 0.524 |
| G110 Control | 2.046 | 2.194 | 0.883 | 0.524 |

Analysis for group to group differences between the $CD4^+$ counts by student t test showed no differences between IEB and the controls $t = 0.67$, $p= 0.52$

b) $CD8^+$ counts Summary statistics.

| Goat No. | mean | median | stdev | SE mean |
|---|---|---|---|---|
| G74 I/T | 0.779 | 0.754 | 0.197 | 0.010 |
| G107 IEB | 0.789 | 0.683 | 0.506 | 0.253 |
| G108 IEB | 1.43 | 1.34 | 0.753 | 0.377 |
| G109 Control | 1.383 | 1.043 | 0.901 | 0.454 |
| G110 control | 2.628 | 2.457 | 1.392 | 0.692 |

Analysis for group to group differences between the $CD8^+$ counts by student t test showed that there were no significant differences $t = -1.53$, $p= 0.16$.

**Appendix B** Table 5c. γδ counts Summary statistics

| Goat No. | mean | median | stdev | SE mean |
|---|---|---|---|---|
| G74 I/T | 0.0103 | 0.111 | 0.046 | 0.023 |
| G107 IEB | 0.149 | 0.151 | 0.074 | 0.036 |
| G108 IEB | 0.734 | 0.683 | 0.463 | 0.232 |
| G109 Control | 0.686 | 0.559 | 0.364 | 0.182 |
| G110 Control | 0.875 | 0.909 | 0.370 | 0.185 |

Analysis for group to group differences between the γ/δ counts of IEB and controls indicated that there were no significant differences $t = -1.70$, $p = 0.11$.
Analysis for group to group differences the monocyte counts of the IEB and control groups showed no significant differences $t = 0.49$, $p = 0.37$.
There also no significant differences between the neutrophil counts of the two groups $t = 0.15$, $p = 0.88$.

**Appendix B Table 6**. Effect of challenge with a virulent homologous culture of *C. ruminantium* on the mean monocyte counts $10^9$/l) in peripheral blood of immunised and control goats.

| | counts & days post challenge | | | |
|---|---|---|---|---|
| group | 0 | 3 | 7 | 10 |
| I/T n=1 | 0.19 | 0.12 | 0.37 | 0.32 |
| IEB n=2 | 0.155 | 0.61 | 0.400 | 1.85 |
| Control n=2 | 0.68 | 0.46 | 1.06 | 2.22 |

**Appendix B Table 7**. Effect of challenge with a virulent homologous culture of *C. ruminantium* on the mean neutrophil counts ($10^9$/l) in peripheral blood of immunised and control goats.

| | counts & days post challenge | | | |
|---|---|---|---|---|
| group | 0 | 3 | 7 | 10 |
| I/T n=1 | 2.46 | 7.78 | 4.44 | 7.02 |
| IEB n=2 | 5.61 | 6.64 | 9.41 | 8.65 |
| Control n=2 | 7.35 | 8.62 | 4.68 | 10.36 |

**Appendix B Table 8a**. Changes in the absolute number ($10^9$/l) of $CD4^+$ T-cells in peripheral blood of the sheep inoculated with 58kDa recombinant Hsp following immunisation and after challenge.

| | | days post-challenge & CD4+ counts | | | |
|---|---|---|---|---|---|
| group | sheep | 0 | 3 | 7 | 12 |
| immunised | S66 | 0.235 | 1.082 | 0.524 | |
| | S69 | 0.838 | 1.807 | 0.647 | 1.143 |
| | S71 | 1.385 | 1.177 | 1.034 | 0.747 |
| | S72 | 1.315 | 2.097 | 0.868 | |
| | S74 | 0.871 | 1.120 | 0.992 | |
| | S76 | 2.045 | 2.708 | 1.561 | |

**Appendix B Table 8b**. Summary data for $CD4^+$ counts (days) of immunised group post challenge.

| day | mean | stdev | sem | median |
|---|---|---|---|---|
| 0 | 1.115 | 0.615 | 0.251 | 1.093 |
| 3 | 1.665 | 0.659 | 0.269 | 1.492 |
| 7 | 0.938 | 0.364 | 0.1485 | 0.930 |

**Appendix B Table 8c**. Summary data for $CD4^+$ counts of immunised group after challenge.

| sheep | mean | stdev | sem | median |
|---|---|---|---|---|
| S66 | 0.6137 | 0.4306 | 0.2486 | 0.5240 |
| S69 | 1.097 | 0.622 | 0.3591 | 0.8380 |
| S71 | 1.199 | 0.177 | 0.102 | 1.177 |
| S72 | 1.427 | 0.622 | 0.359 | 1.315 |
| S74 | 0.994 | 0.124 | 0.072 | 0.992 |
| S76 | 2.105 | 0.576 | 0.333 | 2.045 |

**Appendix B Table 9a**. Changes in the absolute number ($10^9$/l) of $CD4^+$ T cells in the peripheral blood of control group before and afterr challenge.

| | | days post challenge & counts | | | |
|---|---|---|---|---|---|
| group | Sheep | 0 | 3 | 7 | 12 |
| control | S67 | 1.487 | 1.614 | 0.813 | 1.597 |
| | S68 | 1.233 | 1.684 | 0.939 | 1.364 |
| | S70 | 0.743 | 0.803 | 0.503 | 0.239 |
| | S73 | 0.827 | 0.997 | 0.213 | |
| | S75 | 1.210 | 1.050 | 0.494 | |
| | S77 | 0.464 | 0.614 | 0.426 | |

**Appendix B Table 9b**. Summary data $CD4^+$ counts ($10^9$) of the control group before and after challenge.

| day | mean | stdev | sem | median |
|---|---|---|---|---|
| 0 | 0.994 | 0.379 | 0.155 | 1.020 |
| 3 | 1.127 | 0.433 | 0.177 | 1.023 |
| 7 | 0.565 | 0.266 | 0.109 | 0.498 |

**Appendix B Table 9c**. Summary data of $CD4^+$ counts ($10^9$) of the control group before and after challenge.

| Sheep | mean | stdev | sem | median |
|---|---|---|---|---|
| S67 | 1.378 | 0.381 | 0.519 | 1.542 |
| S68 | 1.305 | 0.309 | 0.154 | 1.299 |
| S70 | 0.575 | 0.257 | 0.129 | 0.623 |
| S73 | 0.679 | 0.412 | 0.238 | 0.827 |
| S75 | 0.918 | 0.376 | 0.217 | 1.050 |
| S77 | 0.501 | 0.099 | 0.057 | 0.464 |

**Appendix B Table 10a.** Changes in the absolute numbers ($10^9$/l) of $CD8^+$ T cells in peripheral blood of sheep before and after challenge.

| group | | days post challenge & counts | | | |
|---|---|---|---|---|---|
| immunised | sheep | 0 | 3 | 7 | 12 |
| | S66 | 0.032 | 0.165 | 0.080 | |
| | S69 | 0.267 | 0.534 | 0.121 | 0.411 |
| | S71 | 0.238 | 0.318 | 0.318 | 0.44 |
| | S72 | 0.395 | 0.262 | 0.287 | |
| | S74 | 0.165 | 0.225 | 0.171 | |
| | S76 | 0.308 | 0.453 | 0.242 | |
| Control | S67 | 0.316 | 0.764 | 0.219 | 1.198 |
| | S68 | 0.382 | 0.31 | 0.293 | 0.675 |
| | S70 | 0.335 | 0.427 | 0.199 | 0.143 |
| | S73 | 0.144 | 0.199 | 0.121 | |
| | S75 | 0.199 | 0.564 | 0.216 | |
| | S77 | 0.099 | 0.144 | 0.101 | |

**Appendix B Table 10b**. Summary data of $CD8^+$counts ($10^9$/l) days before and afterchallenge in the immunised group.

| days | mean | stdev. | sem | median |
|---|---|---|---|---|
| day 0 | 0.234 | 0.125 | 0.051 | 0.252 |
| day3 | 0.326 | 0.141 | 0.058 | 0.290 |
| day7 | 0.203 | 0.094 | 0.039 | 0.206 |

**Appendix B Table 10c**. Summary of $CD8^+$counts ($10^9$/l) of individual animals in immunised group.

| sheep | mean | stdev | sem | median |
|---|---|---|---|---|
| S66 | 0.092 | 0.067 | 0.039 | 0.080 |
| S69 | 0.307 | 0.210 | 0.121 | 0.267 |
| S71 | 0.291 | 0.046 | 0.027 | 0.318 |
| S72 | 0.315 | 0.071 | 0.048 | 0.287 |
| S74 | 0.187 | 0.033 | 0.019 | 0.171 |
| S76 | 0.334 | 0.108 | 0.062 | 0.308 |

**Appendix B Table 11a.** Summary data of $CD8^+$counts ($10^9$/l) of days before and afterchallenge in the control group.

| group | Means | stdev | SE | median |
|---|---|---|---|---|
| day 0 | 0.246 | 0.114 | 0.047 | 0.258 |
| day 3 | 0.372 | 0.258 | 0.105 | 0.313 |
| day 7 | 0.192 | 0.071 | 0.029 | 0.208 |

**Appendix B Table 11b.** Summary data of $CD8^+$ counts ($10^9$/l) of individual animals in control group.

| group | mean | SD deviation | SE of mean | median |
|---|---|---|---|---|
| S67 | 0.433 | 0.290 | 0.168 | 0.316 |
| S68 | 0.270 | 0.128 | 0.073 | 0.293 |
| S70 | 0.320 | 0.115 | 0.066 | 0.335 |
| S73 | 0.155 | 0.040 | 0.0234 | 0.144 |
| S75 | 0.326 | 0.206 | 0.120 | 0.216 |
| S77 | 0.115 | 0.025 | 0.015 | 0.010 |

**Appendix B Table 12.** Ratios of $CD4^+$:$CD8^+$ T cells in peripheral blood of immunised and control sheep with virulent culture of *C. ruminantium* (Welgevonden).

| Group/Sheep | ratios/days post challenge | | |
|---|---|---|---|
| immunised | day 3 | day 7 | day 12 |
| S66 | 7:1 | 6:1 | 5:1 |
| S69 | 4:1 | 6:1 | 3:1 |
| S71 | 4:1 | 3:1 | 2:1 |
| S72 | 8:1 | 3:1 | |
| S74 | 5:1 | 6:1 | |
| S76 | 6:1 | 6:1 | |
| control | | | |
| S67 | 2:1 | 4:1 | 1.3:1 |
| S68 | 5:1 | 3:1 | 2:1 |
| S70 | 2:1 | 2:1 | 2:1 |
| S73 | 5:1 | 9:1 | |
| S75 | 3:1 | 2:1 | |
| S77 | 4:1 | 4:1 | |

The ratios of CD4:CD8 show that $CD4^+$ remained higher than $CD8^+$ throughout challenge period.

**Appendix B Table 13a**. Changes in the absolute number ($10^9$/l) of γ/δ T-cells in peripheral blood of sheep before and after challenge with virulent homologous culture *C. ruminantium* (Welgevonden).

| | | days after challenge & counts | | | |
|---|---|---|---|---|---|
| group | Sheep | day 0 | day 3 | day 7 | day 12 |
| immunised | S66 | 0.0012 | 1.169 | 0.412 | |
| | S69 | 0.0011 | 0.964 | 0.264 | 0.160 |
| | S71 | 0.0 | 2.613 | 0.291 | 0.283 |
| | S72 | 0.006 | 1.360 | 0.5.3 | |
| | S74 | 0.003 | 0.842 | 0.614 | |
| | S76 | 0.035 | 1.287 | 0.166 | |
| control | S67 | 0.005 | 1.02 | 0.499 | 0.365 |
| | S68 | 0.005 | 1.237 | 0.270 | 0.363 |
| | S70 | 0.002 | 0.429 | 0.108 | 0.081 |
| | S73 | 0.013 | 1.280 | 0.378 | |
| | S75 | 0.004 | 1.586 | 0.486 | |
| | S77 | 0.005 | 0.498 | 0.171 | |

**Appendix B Table 13b**. Anova two way analysis of T cell counts of sheep immunised with recombinant 58 kDa Hsp and controls.

| Source of variation | SS | DF | V | F20 | Tabular | | significance |
|---|---|---|---|---|---|---|---|
| Between sets | 3.088 | 3 | | | F05 | F01 | |
| Between days p.c. | 3.047 | 1 | 3.047 | 40.0 | 4.35 | 8.09 | VS |
| Between treatments | 0.038 | 1 | 0.030 | 0.050 | '' | '' | NS |
| Interactions | 0.003 | 1 | 0.003 | 0.04 | ''.. | '' | NS |
| Within sets | 1.520 | 20 | 0.076 | | | | NS |

**Key**
VS = very significant, NS = not significant

**Appendix B Table 14a.** Changes in the absolute number ($10^9$/l) of monocytes in peripheral blood of sheep before and after challenge in immunised group with virulent homologous culture of *C. ruminantium* (Welgevonden).

| group | Sheep | Days post challenge & counts | | | |
|---|---|---|---|---|---|
| | | 0 | 3 | 7 | 12 |
| immunised | S66 | 4.80 | 5.30 | 3.00 | |
| | S69 | 5.20 | 8.70 | 4.70 | 5.70 |
| | S71 | 11.80 | 12.40 | 8.00 | 6.70 |
| | S72 | 15.10 | 13.60 | 13.80 | |
| | S74 | 6.30 | 8.70 | 5.60 | |
| | S76 | 9.50 | 11.30 | 8.90 | |
| control | S67 | 8.70 | 12.70 | 6.30 | 7.30 |
| | S68 | 6.80 | 10.50 | 6.70 | 7.90 |
| | S70 | 6.30 | 6.50 | 6.00 | 3.60 |
| | S73 | 7.30 | 8.90 | 6.70 | |
| | S75 | 8.10 | 8.90 | 7.10 | |
| | S77 | 5.70 | 4.70 | 5.20 | |

**Appendix B Table 14b**. Summary data of monocyte counts ($10^9$/l) days before and after challenge in the immunised group.

| days | mean | stdev. | sem | median |
|---|---|---|---|---|
| 0 | 8.783 | 4.108 | 1.677 | 7.900 |
| 3 | 10.000 | 3.029 | 1.237 | 10.000 |
| 7 | 7.330 | 3.832 | 1.565 | 6.800 |

**Appendix B Table 14c**. Summary of monocyte counts ($10^9$/l) of individual animals in immunised group.

| sheep | mean | stdev | sem | median |
|---|---|---|---|---|
| S66 | 4.367 | 1.210 | 0.698 | 4.800 |
| S69 | 6.200 | 2.179 | 1.258 | 5.200 |
| S71 | 10.733 | 2.386 | 1.378 | 11.800 |
| S72 | 14.167 | 0.815 | 0.470 | 13.800 |
| S74 | 6.867 | 1.626 | 0.939 | 6.300 |
| S76 | 9.900 | 1.249 | 0.721 | 9.500 |

**Appendix B Table 14d**. Summary data monocyte counts ($10^9$/l) of days in the control group.

| days | Means | stdev | sem | median |
|---|---|---|---|---|
| 0 | 7.150 | 1.120 | 0.457 | 7.050 |
| 3 | 8.700 | 2.834 | 1.157 | 8.900 |
| 7 | 6.333 | 0.671 | 0.274 | 6.500 |

**Appendix B Table 14e**. Summary data monocyte counts of sheep in the control group

| sheep | mean | std. deviation | sem | median |
|---|---|---|---|---|
| S67 | 9.233 | 3.233 | 1.867 | 8.700 |
| S68 | 8.000 | 2.166 | 1.250 | 6.800 |
| S70 | 6.267 | 0.217 | 0.145 | 6.300 |
| S73 | 7.633 | 1.137 | 0.657 | 7.300 |
| S75 | 8.033 | 0.902 | 0.521 | 8.100 |
| S77 | 5.200 | 0.500 | 0.289 | 5.300 |

**Appendix B Table 15a**. Changes in the absoltue number ($10^9$/l) of neutrophil in peripheral blood of immunised and control sheep before and after challenge with virulent homologous culture of *C. ruminantium* (Welgevonden).

| group | Sheep | days post challenge & counts | | | |
|---|---|---|---|---|---|
| | | 0 | 3 | 7 | 12 |
| immunised | S66 | 3.396 | 1.060 | 1.521 | |
| | S69 | 2.704 | 3.393 | 2.726 | |
| | S71 | 3.422 | 4.464 | 0.080 | |
| | S72 | 4.832 | 6.120 | 6.072 | |
| | S74 | 2.646 | 4.490 | 1.736 | |
| | S76 | 2.660 | 3.204 | 3.204 | |
| control | S67 | 3.741 | 5.715 | 2.52 | 2.92 |
| | S68 | 1.904 | 2.205 | 2.412 | 3.239 |
| | S70 | 3.402 | 3.51 | 3.6 | 2.52 |
| | S73 | 2.482 | 4.005 | 2.546 | |
| | S75 | 1.458 | 2.581 | 1.207 | |
| | S77 | 1.596 | 1.504 | 2.08 | |

**Appendix B Table 15b**. Summary data for neutrophil counts ($10^9$/l) of days in the immunised group before and after challenge.

| days | mean | stdev. | sem | median |
|---|---|---|---|---|
| 0 | 3.277 | 0.844 | 0.345 | 0.305 |
| 3 | 3.789 | 1.693 | 0.691 | 3.928 |
| 7 | 2.557 | 2.034 | 0.830 | 2.231 |

**Appendix B Table 15c**. Summary of neutrophill counts ($10^9$/l) in peripheral blood of individual animals in immunised group after challenge

| sheep | mean | stdev | sem | median |
|---|---|---|---|---|
| S66 | 1.992 | 1.237 | 0.714 | 1.521 |
| S69 | 2.941 | 0.392 | 0.226 | 2.726 |
| S71 | 2.655 | 2.290 | 1.322 | 3.422 |
| S72 | 5.675 | 0.730 | 0.423 | 6.072 |
| S74 | 2.957 | 1.403 | 0.801 | 2.646 |
| S76 | 3.023 | 0.314 | 0.181 | 3.204 |

**Appendix B Table 15d**. Summary data neutrophil counts ($10^9$/l) of days in the control group before and after challenge.

| days | means | stdev | std error of mean | median |
|---|---|---|---|---|
| 0 | 2.431 | 0.957 | 0.391 | 2.193 |
| 3 | 3.253 | 1.503 | 0.614 | 3.045 |
| 7 | 2.394 | 0.775 | 0.316 | 2.466 |

**Appendix B Table 15e.** Summary data neutrophil counts ($10^9$/l) in peripheral blood of sheep in the control group before and after challenge.

| sheep | mean | std. deviation | sem | median |
|---|---|---|---|---|
| S67 | 3.992 | 1.612 | 0.931 | 3.741 |
| S68 | 2.1740 | 0.255 | 0.148 | 2.205 |
| S70 | 3.504 | 0.099 | 0.057 | 3.510 |
| S73 | 3.011 | 0.861 | 0.497 | 2.546 |
| S75 | 1.749 | 0.732 | 0.422 | 1.458 |
| S77 | 1.727 | 0.309 | 0.179 | 1579 |

**Appendix B Table 16a**. Serological responses of sheep immunised with 58kDa Hsp recombinant antigen of *C. ruminantium*. Median ELISA absorbance values at 450 nm.

| | days post challenge and absorbance values | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| group | 0 | 7 | 14 | 21 | 28 | 34 | 64 | 82 |
| S66 | 0.011 | 0.03 | 0.028 | 0.048 | 0.042 | 0.115 | 0.03 | 0.084 |
| S69 | 0.011 | 0.092 | 0.221 | 0.071 | 0.31 | 0.398 | 0.272 | 0.322 |
| S71 | 0.053 | 0.069 | 0.083 | 0.183 | 0.207 | 0.383 | 0.192 | 0.154 |
| S72 | 0.033 | 0.082 | 0.116 | 0.137 | 0.197 | 0.294 | 0.117 | 0.313 |
| S74 | 0.015 | 0.055 | 0.053 | 0.105 | 0.119 | 0.175 | 0.05 | 0.198 |
| S76 | 0.004 | 0.02 | 0.035 | 0.032 | 0.102 | 0.088 | 0.07 | 0.163 |

**Appendix B Table 16b**. Serological responses of control sheep to 35 kDa antigen of *C. ruminantium* Mean ELISA absorbance values (450 nm)

| group | days post challenge & absorbance values (450 nm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Controls | 0 | 7 | 14 | 21 | 28 | 64 | 82 |
| S67 | 0.022 | 0.063 | 0.132 | 0.216 | 0.218 | 0.178 | |
| S68 | 0.01 | 0.035 | 0.070 | 0.076 | 0.072 | 0.060 | |
| S70 | 0.026 | 0.069 | 0.100 | 0.167 | 0.184 | 0.143 | 0.13 |
| S73 | 0.007 | 0.017 | 0.026 | 0.046 | 0.076 | 0.080 | 0.06 |
| S75 | 0.017 | 0.046 | 0.065 | 0.094 | 0.072 | 0.020 | 0.08 |

**Appendix B Table 17.** IgG1 and IgG2 responses of sheep immunised with a recombinant 58kDa antigen to 35kDa subclone.

| | absorbance values (450 nm) of isotypes at different days | | | | | |
|---|---|---|---|---|---|---|
| | Day34 PI | | day 64 PI | | day 143 PI | |
| Sheep | IgG1 | IgG2 | IgG1 | IgG2 | IgG1 | IgG2 |
| S66 | 0.106 | 0.00 | 0.047 | 0.00 | 0.068 | 0.00 |
| S69 | 0.427 | 0.292 | 0.532 | 0.237 | 0.527 | 0.146 |
| S71 | 0.775 | 0.149 | 0.188 | 0.00 | 0.267 | 0.00 |
| S72 | 0.248 | 0.143 | 0.089 | 0.017 | 0.221 | 0.00 |
| S74 | 0.094 | 0.00 | 0.047 | 0.00 | 0.067 | 0.00 |
| S76 | 0.087 | 0.00 | 0.062 | 0.00 | 0.158 | 0.00 |

**Appendix B Table 18**. Proliferative responses *in vitro* of PBMC of sheep stimulated with 15 μg/ml bacterial lysates containing recombinant 58 kDa Hsp (Cr9.4) of *C. ruminantium* or plasmid control (pBluescript).

| | Sheep | antigens/absorbance values at 570 nm | | |
|---|---|---|---|---|
| immunised | S71 | concentration | Cr9.4 | pBS |
| | | 0 | 0.148 | 0.19 |
| | | 1 | 0.265 | 0.21 |
| | | 2.5 | 0.312 | 0.163 |
| | | 5 | 0.236 | 0.169 |
| | | 10 | 0.296 | 0.168 |
| | | 15 | 0.673 | 0.18 |
| | | concentration. | antigen | |
| | S74 | | Cr9.4 | pBS |
| | | 0 | 0.133 | 0.123 |
| | | 1 | 0.124 | 0.13 |
| | | 2.5 | 0.234 | 0.101 |
| | | 5 | 0.228 | 0.109 |
| | | 10 | 0.376 | 0.115 |
| | | 15 | 0.738 | 0.129 |
| | | | Cr9.4 | pBS |
| | S76 | | | |
| | | 0 | 0.113 | 0.115 |
| | | 1 | 0.185 | 0.111 |
| | | 2.5 | 0.213 | 0.112 |
| | | 5 | 0.256 | 0.111 |
| | | 10 | 0.422 | 0.135 |
| | | 15 | 0.639 | 0.11 |
| control | S67 | | Cr9.4 | pBS |
| | | 0 | 0.132 | 0.145 |
| | | 1 | 0.19 | 0.145 |
| | | 2.5 | 0.289 | 0.179 |
| | | 5 | 0.254 | 0.165 |
| | | 10 | 0.325 | 0.165 |
| | | 15 | 0.723 | 0.16 |

Cr9.4 = recombinant 58 kDa Hsp (GroEL), pBS = plasmid pBluescript.
OD values are means of two tests

**Appendix B Table 19a**. $IgG_1$ and $IgG_2$ responses of sheep after live infection with *C. ruminantium* (Nonile and Mara stocks).

| | ELISA mean absorbance values of isotypes and days serum was collected | | | |
|---|---|---|---|---|
| | Day 14 PI | | Day 28 PI | |
| Sheep | $IgG_1$ | $IgG_2$ | $IgG_1$ | $IgG_2$ |
| S2416 | 0.00 | 0.00 | 0.013 | 0.00 |
| S2455 | 0.14 | 0.001 | 0.605 | 0.000 |
| S2347 | 0.006 | 0.00 | 0.045 | 0.000 |

**Appendix B Table 19b**. $IgG_1$ and $IgG_2$ responses of goats following immunisation with inactivated EB's (day 34 PI) after challenge (day 28 PC) of the goats with a virulent homologous stock of *C.ruminantium* (Welgevonden).

| | $IgG_1$ day 34 PI | | | $IgG_2$ day 34 PI | | |
|---|---|---|---|---|---|---|
| Goat Nos. | Mean OD | ±Sem | Stdev | Mean OD | ±Sem | Stdev |
| G476 | 0.280 | 0.005 | 0.006 | 0.061 | 0.003 | 0.004 |
| G601 | 0.207 | 0.008 | 0.012 | 0.142 | 0.009 | 0.013 |
| G614 | 0.232 | 0.001 | 0.001 | 0.100 | 0.004 | 0.006 |
| G668 | 0.111 | 0.009 | 0.013 | 0.088 | 0.019 | 0.027 |

| | day 28 PC $IgG_1$ | | | day 28 PC $IgG_2$ & ODs | | |
|---|---|---|---|---|---|---|
| Goat Nos. | Mean OD | ±Sem | Stdev | Mean OD | ±Sem | Stdev |
| G476 | 0.970 | 0.051 | 0.072 | 0.418 | 0.051 | 0.071 |
| G601 | 0.849 | 0.052 | 0.074 | 1.067 | 0.008 | 0.011 |
| G614 | 0.625 | 0.141 | 0.199 | 1.031 | 0.022 | 0.030 |
| G668 | 0.013 | 0.029 | 0.040 | 0.42 | 0.006 | 0.008 |

**Appendix B Table 19c.** IgG1 and IgG2 responses of South African cattle after live infection (infection/treatment) with 3 stocks of *C. ruminantium*.

| No. | $IgG_1$ OD1 | OD2 | mean OD | ±Sem | SD |
|---|---|---|---|---|---|
| B7 | 0.087 | 0.112 | 0.100 | 0.012 | 0.018 |
| B9 | 0.605 | 0.576 | 0.591 | 0.014 | 0.021 |
| B10 | 0.729 | 0.831 | 0.780 | 0.051 | 0.072 |
| B25 | 0.328 | 0.332 | 0.330 | 0.002 | 0.003 |
| B60 | 0.044 | 0.041 | 0.043 | 0.002 | 0.002 |
| B61 | 0.308 | 0.32 | 0.314 | 0.006 | 0.008 |
| B155 | 0.24 | 0.233 | 0.237 | 0.004 | 0.005 |
| B196 | 0.94 | 1.003 | 0.972 | 0.032 | 0.045 |
| B199 | 1.714 | 1.778 | 1.746 | 0.032 | 0.045 |
| B200 | 0.062 | 0.036 | 0.049 | 0.013 | 0.018 |
| B201 | 1.641 | 1.612 | 1.627 | 0.014 | 0.021 |
| B202 | 0.83 | 0.828 | 0.829 | 0.001 | 0.001 |
| B203 | 0.696 | 0.833 | 0.765 | 0.069 | 0.097 |
| B204 | 0.443 | 0.454 | 0.449 | 0.006 | 0.008 |
| B205 | 0.932 | 0.957 | 0.945 | 0.012 | 0.018 |
| B206 | 0.192 | 0.194 | 0.193 | 0.001 | 0.001 |
| B211 | 0.335 | 0.318 | 0.327 | 0.008 | 0.012 |
| positive | 0.493 | 0.418 | 0.456 | 0.037 | 0.053 |
| negative | 0.014 | 0.017 | 0.016 | 0.002 | 0.002 |

| No. | $IgG_2$ OD1 | OD2 | mean OD | ±Sem | SD |
|---|---|---|---|---|---|
| B7 | 0.102 | 0.101 | 0.10 | 0.00 | 0.00 |
| B9 | 0.068 | 0.09 | 0.08 | 0.01 | 0.02 |
| B10 | 0.155 | 0.146 | 0.15 | 0.00 | 0.01 |
| B25 | 0.024 | 0.018 | 0.02 | 0.00 | 0.00 |
| B60 | 0.032 | 0.035 | 0.03 | 0.00 | 0.00 |
| B61 | 0.131 | 0.124 | 0.13 | 0.00 | 0.00 |
| B155 | 0.197 | 0.203 | 0.20 | 0.00 | 0.00 |
| B196 | 0.331 | 0.345 | 0.34 | 0.01 | 0.01 |
| B199 | 0.49 | 0.567 | 0.53 | 0.04 | 0.05 |
| B200 | 0.012 | 0.144 | 0.08 | 0.07 | 0.09 |
| B201 | 0.402 | 0.449 | 0.43 | 0.02 | 0.03 |
| B202 | 0.054 | 0.059 | 0.06 | 0.00 | 0.00 |
| B203 | 0.112 | 0.139 | 0.13 | 0.01 | 0.02 |
| B204 | 0.008 | 0.007 | 0.01 | 0.00 | 0.00 |
| B205 | 0.084 | 0.067 | 0.08 | 0.01 | 0.01 |
| B206 | 0.064 | 0.059 | 0.06 | 0.00 | 0.00 |
| B211 | 0.031 | 0.031 | 0.03 | 0.00 | 0.00 |
| positive | 0.041 | 0.048 | 0.04 | 0.00 | 0.00 |
| negative | 0.007 | 0.013 | 0.01 | 0.00 | 0.00 |

**Appendix B**: **Table. 20**. Mortality of mice immunised with recombinant 58 kDa Hsp and controls after challenge with *C. ruminantium* (Welgevonden).

| Group | Antig.+.Adj | type | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| A | Cr9.4+FCA | Lysate | days | 7 | 8 | 9 | 11 | | | |
| | | | No. mice | 1 | 4 | 2 | 1 | | | |
| B | Cr9.4+FIA | " | days | 8 | 9 | | | | | |
| | | | No. mice | 2 | 7 | | | | | |
| C | pBS+FCA | " | days | 7 | 8 | 10 | 11 | 12 | 13 | 16 |
| | | | No. mice | 1 | 2 | 2 | 2 | 1 | 1 | 1 |
| D | pBS+FIA | " | days | 8 | 9 | 11 | | | | |
| | | | No. mice | 1 | 3 | 6 | | | | |
| E | FCA | | days | 10 | 11 | | | | | |
| | | | No. mice | 2 | 3 | | | | | |
| F | FIA | | days | 9 | 10 | 11 | | | | |
| | | | No. mice | 2 | 1 | 2 | | | | |
| G | Cr9.4 | Live | days | 7 | 8 | 9 | 10 | | | |
| | | | No. mice | 2 | 2 | 3 | 1 | | | |
| H | pBS | Live | days | 7 | 8 | 10 | | | | |
| | | | No. mice | 5 | 4 | 1 | | | | |

**Key**

Adj = adjuvant
Cr9.4 = recombinant 58 kDa Hsp (GroEL), pBS = plasmid pBluescript
FCA= Freunds complete adjuvant, FIA = Incomplete Freund adjuvant.

**Appendix B Table 21a**. Rectal temperature reactions (°C) of sheep before and after challenge with virulent culture of *C. ruminantium* (Welgevonden).

| | sheep & temperatures of the immunised group (°C) | | | | | |
|---|---|---|---|---|---|---|
| Days | S66 | S69 | S71 | S72 | S74 | S76 |
| -2 | 40.1 | 40.0 | 40.3 | 40 | 40.2 | 40 |
| -1 | 40.0 | 39.9 | 40 | 40.1 | 40.1 | 40 |
| 0 | 39.8 | 40.1 | 40.1 | 40.2 | 40 | 40.2 |
| 1 | 39.7 | 39.8 | 40.1 | 40.2 | 39.9 | 39.9 |
| 2 | 39.8 | 39.9 | 39.14 | 40.4 | 40.5 | 39.9 |
| 3 | 39.3 | 39.0 | 39.6 | 40.3 | 39.8 | 39.8 |
| 4 | 39.7 | 39.4 | 39.9 | 40.6 | 40.1 | 40.3 |
| 5 | 39.7 | 39.7 | 39.8 | 40.0 | 40.1 | 40.1 |
| 6 | 40.1 | 39.4 | 40.9 | 39.8 | 39.9 | 41.8 |
| 7 | 40.9 | 40.7 | 41.1 | 41.1 | 40.8 | 40.8 |
| 8 | 41.4 | 41.2 | 42.0 | 41.6 | 40.8 | 41.6 |
| 9 | 42.2 | 41.4 | 42.0 | 41.7 | 41.7 | 41.6 |
| 10 | 41.6 | 41.3 | 42.3 | 42.0 | 41.5 | 41.7 |
| 11 | 42.0 | 41.9 | 41.9 | 42.0 | | 42.2 |
| 12 | 41.2 | 41.5 | 42.0 | | | |
| 13 | | 42.0 | | | | |
| 14 | | 42.2 | | | | |
| 15 | | 42.1 | | | | |

**Appendix B Table 21b.** Mean daily rectal temperatures (°C) of immunised sheep after and after challenge with virulent culture of *C. ruminantium* (Welgevonden).

| Day | Mean Temp. | ±Sem | Medians | SD |
|---|---|---|---|---|
| -2 | 40.1 | 0.052 | 40.1 | 0.194 |
| -1 | 40.0 | 0.031 | 40.0 | 0.261 |
| 0 | 40.1 | 0.061 | 40.1 | 0.197 |
| 1 | 39.9 | 0.076 | 39.9 | 0.138 |
| 2 | 39.9 | 0.119 | 39.9 | 0.276 |
| 3 | 40.0 | 0.184 | 39.7 | 0.376 |
| 4 | 39.9 | 0.175 | 40.0 | 0.223 |
| 5 | 40.3 | 0.077 | 39.9 | 0.207 |
| 6 | 40.9 | 0.359 | 40.0 | 0.819 |
| 7 | 41.4 | 0.068 | 40.9 | 0.794 |
| 8 | 41.8 | 0.167 | 41.5 | 0.554 |
| 9 | 41.7 | 0.117 | 41.7 | 0.274 |
| 10 | 42.0 | 0.148 | 41.7 | 0.358 |
| 11 | 42.0 | 0.055 | 42.0 | 0.223 |
| 12 | 42.2 | 0.233 | 41.5 | 0.603 |
| 13 | 42.1 | | 42.0 | |
| 14 | | | 42.2 | |
| 15 | | | 42.1 | |

**Appendix B Table 21c.** Rectal temperature (°C) of the controls before and after challenge with virulent culture of *C. ruminantium* (Welgevonden).

| | sheep & temperatures of the control group in °C | | | | | |
|---|---|---|---|---|---|---|
| Days | S67 | S68 | S70 | S73 | S75 | S77 |
| -2 | 39.9 | 40.2 | 40.2 | 39.8 | 40.1 | 40.3 |
| -1 | 39.6 | 39.7 | 39.9 | 39.8 | 40.1 | 40.3 |
| 0 | 39.7 | 39.9 | 39.9 | 39.9 | 40 | 40.3 |
| 1 | 39.9 | 40.2 | 39.9 | 39.8 | 39.9 | 40 |
| 2 | 39.9 | 39.9 | 39.9 | 40.2 | 40.1 | 40.6 |
| 3 | 39.2 | 39.3 | 39.8 | 40.2 | 39.7 | 39.9 |
| 4 | 39.6 | 39.7 | 40 | 40.2 | 40 | 40 |
| 5 | 39.7 | 39.7 | 39.9 | 40.2 | 40 | 40.1 |
| 6 | 39.8 | 39.5 | 40 | 40.2 | 39.3 | 41.6 |
| 7 | 39.7 | 39.8 | 41.3 | 41.1 | 40.8 | 41.6 |
| | 40.1 | 41.2 | 41.8 | 41.1 | 40.9 | 41.2 |
| 9 | 41.2 | 41.3 | 41.9 | 41.6 | 41.5 | 41.2 |
| 10 | 41.6 | 41 | 42 | 41.5 | 41.2 | 41.7 |
| 11 | 42.2 | 42.1 | 41.7 | 41.7 | 41.9 | 41.7 |
| 12 | 42.5 | 41.3 | 42 | | | |
| 13 | 41.1 | | | | | |

**Appendix B Table 21d**. Mean daily rectal temperatures ($^{o}$C) of control sheep before and after challenge with virulent culture of *C. ruminantium* (Welgevonden).

| Day | Mean Temp | ±Sem | Medians | SD |
|---|---|---|---|---|
| -2 | 40.1 | 0.079 | 40.2 | 0.194 |
| -1 | 39.9 | 0.106 | 39.9 | 0.261 |
| 0 | 40.0 | 0.081 | 39.9 | 0.197 |
| 1 | 40.0 | 0.056 | 39.9 | 0.138 |
| 2 | 40.1 | 0.113 | 40.0 | 0.276 |
| 3 | 39.7 | 0.154 | 39.8 | 0.376 |
| 4 | 39.9 | 0.091 | 40.0 | 0.223 |
| 5 | 39.9 | 0.084 | 40.0 | 0.207 |
| 6 | 40.1 | 0.084 | 39.9 | 0.819 |
| 7 | 40.7 | 0.334 | 41.0 | 0.794 |
| 8 | 41.1 | 0.324 | 41.2 | 0.554 |
| 9 | 41.5 | 0.226 | 41.4 | 0.274 |
| 10 | 41.5 | 0.112 | 41.6 | 0.358 |
| 11 | 41.9 | 0.146 | 41.8 | 0.223 |
| 12 | 41.9 | 0.091 | 42.0 | 0.603 |
| 13 | 41.1 | 0.348 | 41.0 | |

**Appendix B. Table 22.** Rectal temperatures (°C)goats before and after challenge with a virulent culture of *C. rumnantium* (Welgevonden).

| | I/T. | IEB Goats | control goats | | |
|---|---|---|---|---|---|
| days post challenge | G74 (I/T) | G107 (IEB) | G108 | G109 | G110 |
| -2 | 40.1 | 39.2 | 40.1 | 40.2 | 39.9 |
| -1 | 39.8 | 39.5 | 39.6 | 39.6 | 39.4 |
| 0 | 39.7 | 39.3 | 40.0 | 39.4 | 39.8 |
| 1 | 39.7 | 39.4 | 40.1 | 39.6 | 39.8 |
| 2 | 39.5 | 39.6 | 40.0 | 39.3 | 39.6 |
| 3 | 39.5 | 39.0 | 40.4 | 40.5 | 39.6 |
| 4 | 39.6 | 39.1 | 39.8 | 40. | 39.6 |
| 5 | 38.8 | 39.4 | 40.0 | 39.8 | 39.6 |
| 6 | 38.6 | 40 | 39.9 | 40.0 | 40.1 |
| 7 | 38.6 | 40.6 | 40.3 | 39.9 | 39.7 |
| 8 | 40.3 | 40.7 | 40.3 | 40.3 | 40.9 |
| 9 | 39.4 | 41.6 | 41.9 | 41.3 | 41.2 |
| 10 | 39.2 | 41.6 | 41.8 | 40.8 | 40.4 |
| 11 | 39.0 | 41.4 | | | |